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(57) Abstract

A method and compounds are provided for inhibiting the synthesis of extracellular matrix proteins. Compounds of the invention comprise oligonucleotides specific for nuclear proto-oncogenes. Preferably, oligonucleotides of the invention are selected from the group consisting of c-*myc* and c-*myb* and are locally administered. The invention finds use in the treatment of a variety of disorders, including sclerotic disorders and restenosis, associated with the inappropriate synthesis of extracellular matrix proteins, particularly collagen.

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# ARTERIOVENOUS AND VENOUS GRAFT TREATMENTS: METHODS AND COMPOSITIONS

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of PCT Application US94/11853 designating the United States, filed October 17, 1994, and herein incorporated by reference, which is a continuation-in-part of application 08/138,637, filed October 15, 1993, and herein incorporated by reference.

## FIELD OF INVENTION

The invention relates generally to oligonucleotides and their use as therapeutic agents, and more particularly to the use oligonucleotide antisense and anti-gene compounds to inhibit the synthesis of extracellular matrix proteins, particularly from fibroblasts and smooth muscle cells.

## INTRODUCTION

Arteriovenous shunts and fistulae provide hemodialysis access sites to the circulatory system for hemodialysis patients. Unfortunately, arteriovenous shunts and fistulae fail over time, which can create serious medical complications for more than 300,000 world-wide hemodialysis patients (Newman, G.E., *in Vascular Disease: Surgical and Interventional Therapy, E. Strandness, Ed. (1994)).* Depending on the study, 12 to 45 percent of arteriovenous shunts and fistulae fail within the first year (Newman, G.E., (1994) cited herein). The major reason for access site failure is progressive venous stenosis (Sedberg *et al.*, *Circulation* 80: 1726-1736 (1989)). Failed or dysfunctional hemodialysis access sites require medical treatment, either surgery to replace the arteriovenous shunt or transcatheter intervention (e.g. balloon angioplasty or stent) to enlarge the venous vessel exiting the shunt or fistulae (Newman, G.E., (1994) cited herein). Consequently, chronic hemodialysis patients with arteriovenous shunt and fistula complications spend approximately 30 days per year in the hospital to maintain the patency of their access sites.

Hemodialysis access sites are thus known as the "Achilles heel" of a hemodialysis patient, even though the construction of an arteriovenous shunt or

fistula is necessary for convenient hemodialysis. Arteriovenous shunts and fistulae are usually constructed as Brescia-Cimino arteriovenous fistulae or polytetrafluoroethylene (PTFE, Gor-Tex™) grafts. In most instances the vein is surgically connected to the artery, either directly, or indirectly via a PTFE graft, in the forearm area, shoulder area or thigh area.

Arteriovenous fistulae are but one example of arteriovenous grafts that progressively become compromised over time. Other types of grafts (i.e., venous grafts) that cause medical conditions requiring treatment include: aortocoronary bypass grafts, carotid grafts, iliofemoral grafts and fermoropopliteal grafts. In these grafts a segment of a vein is placed in the arterial system. One of the predominate medical conditions affecting arteriovenous grafts of all types, or venous grafts placed in the arterial system, is the decrease in the interior diameter of the vessel over time. This usually occurs in the vein exiting, or forming part of the body of, the graft (referred to herein as the "vein portion" of the graft). It is believed that exposure of a vein to high, arterial pressure changes the morphology of the vein; notably the interior diameter of the vein portion of the graft decreases in diameter. Grafts also suffer from thrombosis and other medical disorders, such as infection.

Prior to the present invention, treatments for arteriovenous and venous grafts were of limited effectiveness and usually required invasive treatment. Efforts have focused on mechanically increasing the interior diameter of the graft in the section with thickened vessel walls, such as in failing hemodialysis access sites. Other methods simply rely on surgical revision of the failed graft site, as in arteriovenous shunt replacement. These types of prior art treatments need considerable physician supervision, often require hospitalization and in the case of some hemodialysis access sites, may ultimately lead to an inability to find a suitable hemodialysis access site, as discussed herein.

Several studies focusing on the morphology of veins in failing grafts, such as arteriovenous grafts and venous grafts (e.g., aortocoronary bypasses) have provided information on the pathomechanism of these disorders. Thin-walled veins respond early to exposure to arterial blood flow and pressure. The vein starts to develop neointima. The response to high arterial pressure and flow

ultimately leads to a significant narrowing of the lumen, causing diminished patency of the vein. Vascular smooth muscle cells, are primarily found in the media and neointima. Fibroblasts are found in the adventitia and perivascular tissue. Fibroblasts contribute to the granulation of the perivascular tissue during wound repair of the vein. In response to injury, both smooth muscle cells and fibroblasts increase matrix protein synthesis in the vessel wall and perivascular tissue. Consequently, the vein becomes narrowed and stiffer due to an increase of vessel wall thickness and an increase in scar formation in the perivascular tissue, which leads to irreversible failure of grafts, as in the case of arteriovenous fistulae or venous grafts.

The inappropriate synthesis of extracellular matrix proteins and/or the synthesis of aberrant forms of such proteins is associated with wide range of deleterious conditions, including many rare heritable diseases as well as more commonly acquired disorders, such a loss of arteriovenous and venous graft patency (Sedberg *et al.*, *Circulation* 80: 1726-1736 (1989)), fibrotic skin disease, pulmonary fibrosis, osteoarthritis, vascular restenosis, and the like, e.g., Weiss and Jayson, Editors, *Collagen in Health and Disease* (Churchill Livingstone, Edinburgh, 1982); Gardner, Editor, *Pathological Basis of the Connective Tissue Diseases* (Lea & Febiger, Philadelphia, 1992). Extracellular matrix consists primarily of collagens, proteoglycans, elastin, and fibronectin.

Many of these conditions are associated with very complex biological responses to physical, chemical, and/or biological insults. Such responses include the proliferation and migration of a variety of cell types and the synthesis of a growth factors that contribute to or modify the response. For example, vascular disorders, such as atherosclerosis and vascular restenosis, are associated with local cell proliferation and migration, as well as the production of several classes of structural proteins and many growth factors, including platelet-derived growth factor (PDGF), basis fibroblastic growth factor, tumor necrosis factor $\alpha$, interleukin-1, prostaglandins, and a variety of proto-oncogenes, e.g., Ross, *Nature*, 362: 801-809 (1993); and Morishita *et al*, *Proc. Natl. Acad. Sci.*, 90:8474-8478 (1993). Unfortunately, the precise role of these factors in the various disease processes is not well understood.

The tremendous economic impact of disorders associated with inappropriate production of extracellular matrix proteins, especially vascular disorders, has served as a strong impetus to develop drugs or other methods of treatment to cure or ameliorate their debilitating effects. In this class of disease, as well as others, where a disease condition is associated with the apparent aberrant expression of a endogenous gene, the use of so called antisense compounds provides many advantages, e.g. Milligan *et al*, *J. Med. Chem.*, 36:1923-1937 (1993); Uhlmann and Peyman, Chemical Reviews, 90:543-584 (1990); Goodchild, *Bioconjugate Chemistry*, 1:165-187 (1990); Crooke, *Ann. Rev. Pharmacol. Toxicol.*, 32:329-376 (1992); Stein *et al*, *Science*, 261:1004-1012 (1993); and the like.

A particularly compelling advantage of the antisense approach is that one need not carry out one or more initial screening steps to identify candidate compounds capable of binding to a therapeutic target. If the aberrant expression of a gene is known to cause a disease for which a drug is sought, then the structures of candidate antisense drugs are determined automatically from the nucleotide sequence of the aberrantly expressed gene. One need only provide an oligonucleotide or an analog thereof capable of forming a stable duplex or triplex with such a gene, or associated target polynucleotide, based on Watson Crick or Hoogsteen binding, respectively. It will be recognized that the invention is not limited to a particular mechanism of action. Such compounds may have additional non-antisense, sequence specific affects that rely on other mechanisms, such as but not limited to, aptameric affects. The specifically bound antisense compound then either renders the respective targets more susceptible to enzymatic degradation, blocks translation or processing, or otherwise blocks or inhibits the function of a target polynucleotide.

It would be highly advantageous if particular genes were identified whose expression were causally related to the synthesis of structural proteins, such as collagen, that contribute to disease conditions. Such identification would immediately lead to the prospect of treatments for a number of disorders associated with the excess synthesis of such proteins by the antisense approach.

## SUMMARY OF THE INVENTION

The invention provides a method of treating disorders associated with the inappropriate synthesis of extracellular matrix proteins, particularly collagen. The method comprises the step of administering to an individual in need of such treatment an effective amount of one or more antisense oligonucleotides specific for nuclear proto-oncogenes. As defined more fully below, an "antisense oligonucleotide specific for a nuclear proto-oncogene" is an oligonucleotide having a sequence (i) capable of forming a stable duplex with a portion of an mRNA transcript of a nuclear proto-oncogene or cell cycling gene, or (ii) capable of forming a stable triplex with a portion of a nuclear proto-oncogene or cell cycling gene. Preferably, the antisense oligonucleotides of the invention form stable duplexes with portions of an mRNA transcript of a nuclear proto-oncogene. More preferably, antisense oligonucleotides of the invention are specific for mRNA transcripts of c-*myc* or c-*myb* proto-oncogenes. Most preferably, antisense oligonucleotides of the invention are specific for mRNA transcripts of the c-*myc* proto-oncogene.

Preferably, c-*myc* antisense oligonucleotides are administered locally to the site at which the inappropriate synthesis of extracellular matrix proteins takes place.

The invention includes a method for preventing failure of a hemodialysis access site and vascular grafts by contacting the hemodialysis access site or vascular graft with an oligonucleotide complementary to nuclear proto-oncogene mRNA, either *ex vivo*, or *in vivo*, in a human. Additionally the invention includes compositions comprised of hemodialysis access sites, vascular grafts or excised human-compatible veins with oligonucleotides of the invention in the components or tissues of such structures.

The invention further includes a method of reducing scar formation in a human tissue by administering a therapeutically effective amount of a proto-oncogene antisense oligonucleotide to the tissue.

The invention also includes a method of inhibiting formation of fibrous connective tissue in a human by administering a therapeutically effective amount of a proto-oncogene antisense oligonucleotide.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 describes an example of an arteriovenous graft before and after arterialization, and a loss in patency of the vein portion of the graft.

FIG. 2a is a photograph of a cross-section of a normal porcine vein stained with Verhoff stain (magnification x 10).

FIG 2b is a cross-section of an arterialized porcine vein stained with Verhoff stain (magnification x 5).

FIG. 3a is a photograph of a cross-section of a normal porcine vein stained with Sirrius red (magnification x 5).

FIG. 3b is a photograph of a cross-section of an arterialized porcine vein stained with Sirrius red (magnification x 5).

FIG. 4a is a photograph of a cross-section of a normal porcine vein stained with antibodies for $\alpha$-actin (magnification x 10).

FIG. 4b is a photograph of a cross-section of an arterialized porcine vein stained with antibodies for $\alpha$-actin (magnification x 5).

FIG. 4c is a photograph of a cross-section of a normal porcine vein stained with antibodies for desmin (magnification x 10).

FIG. 5a is a photograph of a cross-section of an arterialized porcine vein stained with antibodies for proliferating cell nuclear antigen (PCNA) (magnification x 25).

FIG. 5b is a photograph of a cross-section of an arterialized porcine vein stained with Verhoff stain (magnification x 25).

FIG. 5c is a photograph of a cross-section of an arterialized porcine vein stained with Sirrius red (magnification x 25).

FIG. 6a is a photograph of a cross-section of a human vein from an aortocoronary graft stained with Verhoff stain (magnification x 5).

FIG. 6b is a photograph of a cross-section of a human human vein from an aortocoronary graft stained with Sirrius red (magnification x 5).

FIG. 6c is a photograph of a cross-section of a human vein from an aortocoronary graft stained with antibodies for $\alpha$-actin (magnification x 5).

FIG. 6d is a photograph of a cross-section of a human vein from an aortocoronary graft stained with antibodies to PCNA (magnification x 50).

FIG. 7a is a photograph of a cross-section of a porcine vein organ culture treated with PDG (Platelet Derived Growth Factor) and stained with antibodies for PCNA (Proliferating Cell Nuclear Antigen) and counter-stained with hematoxilyn and eosin (magnification x 50).

FIG. 7b is a photograph of a cross-section of a porcine vein organ culture without PDGF treatment stained with antibodies for PCNA and counter-stained with hematoxilyn and eosin stains (magnification x 25).

FIG. 7c is a photograph of a cross-section of a porcine arterialized vein treated with PDGF and oligonucleotide (SEQ ID No:1) and stained with antibodies with PCNA and counter-stained with hematoxilyn and eosin stains (magnification x 50).

FIG. 8a is a photograph of a cross-section of a human vein organ culture exposed to fluorescein end-labeled oligonucleotide (SEQ ID No:1) for two seconds (magnification x 25).

FIG. 8b is a photograph of a cross-section of a human vein organ culture exposed to fluorescein end-labeled oligonucleotide (SEQ ID No:1) for 30 minutes (magnification x 25).

FIG. 8c is a photograph of a cross-section of a human vein organ culture incubated with a fluorescein end-labeled oligonucleotide (SEQ ID No:1) for one hour (magnification x 25).

FIG. 8d is a photograph of a cross-section of a human vein organ culture incubated with fluorescein end-labeled oligonucleotide (SEQ ID No:1) for two hours (magnification x 25).

FIG. 9a is a photograph of a cross-section of a porcine vein perivascularly injected with fluorescein end-labeled oligonucleotide (SEQ ID No:1) for two hours (magnification x 50).

FIG. 9b is a photograph of a cross-section of a porcine vein percutaneously injected with fluorescein end-labeled oligonucleotide (SEQ ID No:1) for thirty minutes (magnification x 50).

FIG. 9c is a photograph of a cross-section of a porcine vein percutaneously administered fluorescein and labeled oligonucleotide (SEQ ID No:1) for two hours post injection (magnification x 50).

FIG. 10 is a photograph of a cross-section of an exemplary control (i.e., which received sense oligomer injection) porcine coronary artery one month following injury.

FIG. 11 is a photograph of a cross-section of an exemplary antisense-treated porcine coronary artery.

FIG. 12 illustrates maximal neointimal area as a function of degree of injury.

FIG. 13 describes the effect of antisense oligonucleotides targeting the translation initiation region of *c-myc* mRNA on extracellular levels of procollagen I, procollagen III and fibronectin in post-confluent human smooth muscle cells. Procollagen I and procollagen III in conditioned medium were determined by Western blot, whereas fibronectin was measured by immunoprecipitation.

FIG. 14 describes the sequence-specific effect of *c-myc* antisense oligonucleotides on type I collagen. Human smooth muscle cells were incubated with different control oligonucleotides (16 $\mu$M) including sense, 4 bp mismatched and scrambled sequences and antisense oligonucleotides (16 $\mu$M) targeting various regions of *c-myc* mRNA for 24 hours. The levels of type I collagen in media were measured after conditioned pepsin digestion using Western blot. *Top panel:* Blots demonstrate collagen $\alpha$ chains. *Lane 1*: no oligonucleotides; *Lane 2*: sense oligonucleotides; *Lane 3*: 4 bp mismatched ODN (referred to as "oligonucleotide"); *Lane 4*: scrambled oligonucleotides; *Lane 5*: antisense targeting the translation initiation regions of *c-myc* mRNA (position 559-573, AS1); *Lane 6*: antisense targeting exon 1 (position 400-419, AS2) and *Lane 7*: antisense targeting translated region in exon 3 (position 1264-1283, AS3). *Lower panel*: Histograms depicting percent change of control values (no oligonucleotides) obtained from densitometric measurements of 3 separate experiments (mean $\pm$ SEM). Mis: 4 bp mismatched oligonucleotides; Scr: scrambled oligonucleotides.

FIG. 15 describes the inhibition of *c-myc* protein by antisense oligonucleotides (AS1). Post-confluent smooth muscle cells were incubated with or without antisense oligonucleotides for 24 hours. The p62 *c-myc* was determined by immunoprecipitation of [$^{35}$S]methionine-labeled nuclear extracts and it is shown on autoradiogram of SDS-polacrylamide gel.

FIG. 16 describes metabolic state of smooth muscle cells following oligonucleotides. Cells were preincubated with or without oligonucleotides (16 $\mu$M) for 4 hours and labeled with [$^{35}$S]methionine for 16 hours. The radiolabeled proteins were measured after TCA precipitation. Antisense oligonucleotides showed no effect on methionine incorporation into proteins demonstrating normal metabolic activity of smooth muscle cells. Data represent dpm/$10^5$ cells (mean $\pm$ SEM of 2 separate experiments), S: sense; MIS: mismatched; SCR: scrambled; AS1: antisense oligonucleotides targeting translation initiation region of *c-myc* mRNA.

FIG. 17 describes the recovery of type I collagen after the removal of *c-myc* antisense oligonucleotides (AS1). Smooth muscle cells were incubated with or without oligonucleotides (16 $\mu$M) for 24 hours. After 3 washes, medium without oligonucleotides was added and collected 24 hours thereafter. Type I collagen was determined by Western blot after pepsin digestion. A complete recovery of type 1 collagen is seen after the removal of *c-myc* antisense oligonucleotides.

FIG. 18 describes the effect of *c-myc* antisense oligonucleotides on pro $\alpha_1$(I) and pro $\alpha_2$(I) mRNA levels. Total RNA was isolated from smooth muscle cells incubated with or without oligonucleotides (16 $\mu$M) for 24 hours and analyzed by Northern blot (10 $\mu$g/lane). Antisense oligonucleotides (AS1) had no effect on procollagen $\alpha_1$(I) and procollagen $\alpha_2$(I) mRNA levels. Experiments carried out at 6 hours after antisense treatment yielded similar results. 7S RNA showed no change.

FIG. 19 describes the extracellular and intracellular [$^{14}$C] proline labeled proteins. Human smooth muscle cells were incubated with or without oligonucleotides for 16 hours, then labeled with [$^{14}$C]proline for an additional 4 hours in serum-free medium. [$^{14}$C]proline labeled proteins were analyzed on polyacrylamide SDS gels. The inhibition of radiolabeled proteins in conditioned media was seen which was accompanied by an increased level of intracellular procollagen $\alpha_1$(I) and $\alpha_2$(I) following *c-myc* antisense treatment.

FIG. 20 describes the hydroxyproline content in antisense treated and control human smooth muscle cells.

FIG. 21 describes the effect of *c-myc* antisense treatment on extracellular Type I collagen levels in human skin fibroblasts.

FIG. 22 describes the intracellular and extracellular procollagen level associated with *c-myc* antisense treated and control human skin fibrolasts.

FIG. 23 describes the thermal stability of intracellular procollagen in human skin fibroblasts. The pepsin-purified procollagen from cells treated with or without oligonucleotides was incubated with trypsin and chymotrypsin at indicated temperatures for 2 minutes and electrophoresed on polyacrylamide SDS gels. A similar melting temperature (i.e, about 41°C) was seen regardless of treatment suggesting a normal triple helical conformation of procollagen $\alpha$ chains after *c-myc* antisense oligonucleotides.

FIG. 24 describes the procollagen $\alpha_1$ (I) mRNA level in *c-myc* antisense oligonucleotide treated and control human skin fibroblasts.

FIG. 25 describes the recovery of type I collagen in *c-myc* antisense treated and control human skin fibroblast 24 hours after treatment with oligonucleotides and exposure to fresh medium without oligonucleotides.

FIG. 26 describes a cross-sectional view of a porcine coronary artery and perivascular tissues. Distribution of *c-myc* antisense oligonucleotides (SEQ ID No:1) was assessed within the vessel wall and in the adjacent perivascular tissues (magnification x 20).

FIG. 27 A-F describe the patterns of *c-myc* antisense (SEQ ID No:1) ODN distribution in the coronary arteries 30 minutes following transcatheter administration. *Panels A and B:* Phase-contrast and fluorescence microscopy photomicrographs of the same section demonstrating transmural localization of fluorescein-labeled oligonucleotides. Antisense oligonucleotides are present in the media, adventitia and perivascular tissues. *Panels C and D:* Phase-contrast and fluorescence microscopy photomicrographs showing subintimal, non-transmural ODN distribution. Oligonucleotides are detectable only in the media. *Panels E and F:* Phase-contrast and fluorescence microscopy photomicrographs exhibiting midwall, non-transmural ODN presence magnification x 62.5.

FIG. 28 A and B describe the persistence of *c-myc* antisense oligonucleotides within the arterial wall 3 days following transcatheter

administration. *Panels A and B:* Phase-contrast and fluorescence microscopy photomicrographs of the same section respectively (magnification x 62.5).

FIG. 29 describes a bar graph depicting vessel-associated and perivascular radioactivities following intramural administration of $^{35}S$-labeled phosphorothioate c-*myc* antisense oligonucleotides. A similar total ODN content is noted at 30 min (open bar; n=6) and at 3 days (closed bar; n=4) after their delivery due to ODN trafficking from the perivascular space toward the vessel wall. Results are presented as mean ± SEM.

FIG. 30 A-D describe the preservation of vascular structures following transcatheter administration of c-*myc* antisense oligonucleotides. *Panel A:* fluorescence-labeled oligonucleotides are clearly visible within the coronary aerial wall at 30 minutes after injection. *Panel B:* adjacent sections demonstrate preserved internal elastic lamina and normal orientation of elastic tissues without disruption (Verhoeff stain). *Panel C:* mild decrease of cytoplasmic staining and nuclear pyknosis was noted at the site of ODN retention (hematoxylin and eosin stain). *Panel D:* section adjacent to the section shown in FIG. 19 demonstrating resolution of the above changes 3 days after delivery of oligonucleotides (hematoxylin and eosin stain, magnification x 50).

FIG. 31 describes the intracellular localization of fluorescein-labeled c-*myc* antisense oligonucleotides assessed by confocal microscopy of vascular smooth muscle cells *in vitro*.

FIG. 32 describes the intracellular localization of fluorescein-label *c-myc* antisense oligonucleotides 30 minutes after intramural delivery of oligonucleotides *in vivo*, nuclear localization is visible in medial smooth muscle cells.

FIG. 33 describes the intracellular localization of fluorescein-label *c-myc* antisense oligonucleotides 30 minutes after intramural delivery of oligonucleotides *in vivo*, nuclear localization is visible in adventitial cells.

## DEFINITIONS

The term "collagen" as used herein refers to a class of molecules which are synthesized from procollagen. Collagen is synthesized from procollagen extracellularly, by the cleavage of N- and C- termini outside the cell. Antibodies to the triple helical region of collagen can recognize the triple helical region in

procollagen as well. Antibodies used herein are either specific for collagen I and procollagen I, or collagen III and procollagen III. Collagen can also be synthesized *in vitro* from procollagen by pepsin digestion.

The term "graft" as used herein refers to a vascular graft, such as a arteriovenous or venous graft.

The term "nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described generally by Scheit, Nucleotide Analogs (John Wiley, New York, 1980). Such analogs include synthetic nucleosides designed to enhance binding properties, e.g. duplex or triplex stability, specificity, or the like.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, $\alpha$- anomeric forms thereof, polyamide nucleic acids, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3-4, to several hundreds of monomeric units. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'->3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, 3'→5' phosphoramidate and the like, as more fully described below.

"Stability" in reference to duplex or triplex formation roughly means how tightly an antisense oligonucleotide binds to its intended target sequence; more precisely, it means the free energy of formation of the duplex or triplex under

physiological conditions. Melting temperature under a standard set of conditions, e.g. as described below, is a convenient measure of duplex and/or triplex stability. Preferably, antisense oligonucleotides of the invention are selected that have melting temperatures of at least 50°C under the standard conditions set forth below; thus, under physiological conditions and the preferred concentrations duplex or triplex formation will be substantially favored over the state in which the antisense oligonucleotide and its target are dissociated. It is understood that a stable duplex or triplex may in some embodiments include mismatches between base pairs and/or among base triplets in the case of triplexes. Such as in the case of SEQ ID No:1 which is a human sequence with a one base mismatch in the last nucleotide with respect to the comparable porcine sequence. Preferably, antisense oligonucleotides of the invention form perfectly matched duplexes and/or triplexes with their target polynucleotides.

The term "synthesis" as used herein in a biological context refers to a process involved in the production of biological molecules, in particular extracellular matrix molecules and extracellular matrix proteins. The term "synthesis" includes processes both inside and outside a cell that result in biological molecules, for example, but not limited to, mRNA transcription, mRNA translation, post-translational protein modification, glycosylation, peptidase cleavage, extracellular peptidase cleavage, intra-cellular peptidase cleavage, protein transport, protein secretion and protein phosphorylation. Preferably the invention inhibits protein translation of extracellular matrix molecules, post-translational protein processing, protein secretion or intracellular peptidase cleavage.

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the use of certain antisense compounds to inhibit the inappropriate synthesis in a tissue of extracellular matrix proteins, particularly collagen, and more particularly type I and type III collagen. The inappropriate and/or excessive synthesis of extracellular matrix proteins can result in medical conditions that exhibit formation of, e.g., unwanted fibrous connective tissue. Such medical conditions include sclerotic disorders, vascular restenosis,

atherosclerosis, atherogenesis, keloid disease, liver cirrhosis, rheumatoid disorders of the joints, loss of arteriovenous and venous graft patency, post-surgical scarring, reconstructive surgery, and the like, generally found in human subjects. A range of tissue types can be the site of such inappropriate synthesis, including vascular smooth muscle cells, endothelial cells, skin and organ fibroblasts, e.g. in keloid disease or posttraumatic or postsurgical scar formation, and synovial cells and other joint components. One embodiment of the invention is particularly useful in preventing the failure or loss of patency of arteriovenous and venous grafts. Another embodiment of the invention is particularly useful in the treatment of restenosis, an acute vascular injury, typically following angioplasty in a human subject. Restenosis is associated with smooth muscle cell migration to the vascular intima, proliferation, and synthesis of extracellular matrix components, e.g. Holmes *et al*, Chapter 12 in Vlietstra et al, Editors, *PTCA* (Davis Company, Philadelphia, 1987).

By administering a therapeutically effective amount of an antisense compound to a tissue having a target polynucleotide the inappropriate (i.e. excessive) synthesis of extracellular protein is inhibited. Target polynucleotides may be single stranded or double stranded DNA or RNA; however, single stranded DNA or RNA targets are preferred. The genomic nucleotide sequence and the mRNA transcripts of nuclear oncogenes of the invention are known in the art and include c-*myc*, c-*myb*, c-fos, N-*myc*, L-*myc*, p53, c-rel, c-ski, c-ets-1, c-ets-2, and the like, e.g. Glover, editor, *Oncogenes* (IRL Press, Oxford, 1989). More particularly, *c-myc* and *c-myb* sequences are described in the following references: c-*myc* proto-oncogene sequences are described in Marcu *et al*, *Ann. Rev. Biochem.*, 61:809-860 (1992); Watt *et al*, *Nature*, 303:725-728 (1983); Battey *et al*, *Cell*, 34:779-787 (1983); and Epstein *et al*, *NTIS publication* PB93-100576; the c-*myb* proto-oncogene is described in Gewirtz *et al*, U.S. patent 5,098,890 and Majello et al, *Proc. Natl. Acad. Sci.* 79:9636-9640 (1986). In addition, proliferating cell nuclear antigen, cyclin ($D_1$ and $B_1$), cdc2 kinase, cdk kinase and E2F can be targetted (herein referred to as "cell-cycle genes"). Preferably, the nuclear proto-oncogenes targeted by antisense oligonucleotides of the invention are c-*myc* and/or c-*myb*.

It is understood that the target to which the c-*myc* antisense oligonucleotides of the invention are directed include allelic forms of the proto-oncogenes. There is substantial guidance in the literature for selecting particular sequences for antisense oligonucleotides given a knowledge of the sequence of the target polynucleotide, e.g. Ulmann *et al* (cited above); Crooke (cited above); and Zamecnik and Stephenson, *Proc. Natl. Acad. Sci*, 75: 280-284 (1974). Preferably, the sequences of c-*myc* antisense compounds are selected so that the G-C content is at least 60%. Preferably sequences of the oligonucleotides are selected from 12 to 15 contiguous bases of SEQ. ID No. 1, 5-7, or 8-11. Preferred mRNA targets include the 5' cap site, tRNA primer binding site, the initiation codon site, the mRNA donor splice site, the mRNA acceptor splice site, and the like, e.g. Goodchild *et al*, U.S. patent 4,806,463.

When selecting antisense oligonucleotides to target the ATG initiation site of human *c-myc* in the second exon, antisense oligonucleotides with consecutive sequences can be administered using oligonucleotides 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 bases in length and selected from a twenty five base region on either side of the ATG site (human sequence from Gazin *et al, E.M.B.O. Journal* 3:2:383-387 (1984) the sequence of which in herein incorporated by reference.):

5' cccgc tccagcagcc tcccgcgacg ATG cccctcaacg ttagcttcac caaca 3'

Preferably antisense oligonucleotides will include the ATG site. Twenty five base regions on either side of SEQ ID No:6 and 7 may be also be used to select oligonucleotides of the size discussed in this paragraph.

Antisense oligonucleotides of the invention may comprise any polymeric compound capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-nucleoside interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Antisense compounds of the invention may also contain pendent groups or moieties, either as part of or separate from the basic repeat unit of the polymer, to enhance specificity, nuclease resistance, delivery, or other property related to efficacy, e.g. cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end capping" with one or more nuclease-resistant linkage groups

such as phosphorothioate, and the like. Sequences of certain representative oligonucleotides useful in this invention are set forth in the Sequence Listing included herewith.

Antisense compounds of the invention include the pharmaceutically acceptable salts thereof, including those of alkaline earths, e.g. sodium or magnesium, ammonium or $NX_4^+$, wherein X is $C_1$-$C_4$ alkyl. Other pharmaceutically acceptable salts include organic carboxylic acids such as acetic, lactic, tartaric, malic, isethionic, lactobionic, and succinic acids; organic sulfonic acids such as methanesulfonic, ethanesulfonic, and benzenesulfonic; and inorganic acids such as hydrochloric, sulfuric, phosphoric, and sulfamic acids. Pharmaceutically acceptable salts of a compound having a hydroxyl group include the anion of such compound in combination with a suitable cation such as $Na^+$, $NH_4^+$, or the like.

Preferably, nuclease resistance is conferred on the antisense compounds of the invention by providing nuclease-resistant internucleosidic linkages. Many such linkages are known in the art, e.g. phosphorothioate: Zon and Geiser, *Anti-Cancer Drug Design*, 6: 539-568 (1991); Stec *et al*, U.S. patent 5,151,510; Hirschbein, U.S. patent 5,166,387; Bergot, U.S. patent 5,183,885; phosphorodithioates: Marshall *et al*, *Science*, 259:1564-1570 (1993); Caurathers and Nielsen, International application PCT/US89/02293; phosphoramidates, e.g.-OP(=O)($NR^1IR^2$)-O- with $R^1$ and $R^2$ hydrogen or $C_1$-$C_3$ alkyl: Jager *et al*, Biochemistry, 27:7237-7246 (1988); Froehler *et al*, International application PCT/US90/03138; 3'→5' phosphoramidates (as described in the PCT Application for Gryaznov *et al.* PCT/US95/03585, the compounds and methods are herein incorporated by reference); peptide nucleic acids: Nielsen *et al*, Anti-cancer Drug Design, 8: 53-63 (1993), International application PCT/EP92/O1220; methylphosphonates: Miller *et al*, U.S. patent 4,507,433, Ts'o *et al*, U.S. patent 4,469,863, Miller *et al*, U.S. patent 4,757,055; and P-chiral linkages of various types, especially phosphorothioates, Stec *et al*, European patent application 92301950.9 and Lesnikowski, Bioorganic Chemistry, 21:127-155 (1993). Additional nuclease linkages include phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, alkylphosphotriester such as methyl- and

ethylphosphotriester, carbonate such as carboxymethyl ester, carbamate, morpholino carbamate, 3'-thioformacetal, silyl such as dialkyl ($C_1$-$C_6$)- or diphenylsilyl, sulfamate ester, and the like. Such linkages and methods for introducing them into oligonucleotides are described in many references, e.g. reviewed generally by Peyman and Ulmann (cited above); Milligan *et al* (cited above); Matteucci *et al*, International application PCT/US91/06855. Preferably, phosphorus analogs of the phosphodiester linkage are employed in the compounds of the invention, such as phosphorothioate, phosphorodithioate, phosphoramidate, or methylphosphonate. More preferably, phosphorothioate is employed as the nuclease resistant linkage. It is understood that in addition to the preferred linkage groups, compounds of the invention may comprise additional modifications, e.g. boronated bases, Spielvogel *et al*, 5,130,302; cholesterol moieties, Shea et al, Nucleic Acids Research, 18:3777-3783 (1990) or Letsinger *et al*, Proc. Natl. Acad. Sci., 86:6553-6556 (1989); 5-propenyl modification of pyrimidines, Froehler *et al*, Tetrahedron Lett., 33: 5307-5310 (1992); and the like.

Preferably, antisense compounds of the invention are synthesized by conventional means on commercially available automated DNA synthesizers, e.g. an Applied Biosystems (Foster City, CA) model 380B, 392 or 394 DNA/RNA synthesizer. Preferably, phosphoramidite chemistry is employed, e.g. as disclosed in the following references: Beaucage and Iyer, Tetrahedron, 48:2223-2311 (1992); Molko *et al*, U.S. patent 4,980,460; Koster *et al*, U.S. patent 4,725,677; Caruthers *et al*, U.S. patents 4,415,732; 4,458,066; and 4,973,679; and the like.

In embodiments where triplex formation is desired, there are constraints on the selection of target sequences. Generally, third strand association via Hoogsteen type of binding is most stable along homopyrimidine-homopurine tracks in a double stranded target. Usually, base triplets form in T-A*T or C-G*C motifs (where "-" indicates Watson-Crick pairing and "*" indicates Hoogsteen type of binding); however, other motifs are also possible. For example, Hoogsteen base pairing permits parallel and antiparallel orientations between the third strand (the Hoogsteen strand) and the purine-rich strand of the duplex to which the third strand binds, depending on conditions and the composition of the strands. There is extensive guidance in the literature for selecting appropriate sequences,

orientation, conditions, nucleoside type (e.g. whether ribose or deoxyribose nucleosides are employed), base modifications (e.g. methylated cytosine, and the like) in order to maximize, or otherwise regulate, triplex stability as desired in particular embodiments, e.g. Roberts *et al*, *Proc. Natl. Acad. Sci.*, 88:9397-9401 (1991); Roberts *et al*, *Science*, 258:1463-1466 (1992); Distefano *et al*, *Proc. Natl. Acad. Sci.*, 90:1179-1183 (1993); Mergny *et al*, *Biochemistry*, 30:9791-9798 (1991); Cheng *et al*, I. *Am. Chem. Soc.*, 114:4465-4474 (1992); Beal and Dervan, *Nucleic Acids Research*, 20:2773-2776 (1992); Beal and Dervan, 1. *Am. Chem. Soc.*, 114:4976-4982 (1992); Giovannangeli *et al*, *Proc. Natl. Acad. Sci.*, 89: 8631-8635 (1992); Moser and Dervan, *Science*, 238:645-650 (1987); McShan *et al*, *J. Biol. Chem.*, 267:5712-5721 (1992); Yoon *et al*, *Proc. Natl. Acad. Sci.*, 89:3840-3844 (1992); Blume *et al*, *Nucleic Acids Research*, 20:1777-1784 (1992); and the like.

The length of the oligonucleotide moieties is sufficiently large to ensure that specific binding will take place only at the desired target polynucleotide and not at other fortuitous sites, as explained in many references, e.g. Rosenberg *et al*, International application PCT/US92/05305; or Szostak *et al*, *Meth. Enzymol.* 68:419-429 (1979). The upper range of the length is determined by several factors, including the inconvenience and expense of synthesizing and purifying oligomers greater than about 30-40 nucleotides in length, the greater tolerance of longer oligonucleotides for mismatches than shorter oligonucleotides, whether modifications to enhance binding or specificity are present, whether duplex or triplex binding is desired, and the like. Usually, antisense compounds of the invention have lengths in the range of about 12 to 60 nucleotides. More preferably, antisense compounds of the invention have lengths in the range of about 15 to 40 nucleotides; and most preferably, they have lengths in the range of about 18 to 30 nucleotides.

Preferably, the thermal stability of the antisense oligonucleotides of the invention are determined by way of melting, or strand dissociation, curves. The temperature of fifty percent strand dissociation is taken as the melting temperature, $T_m$, which, in turn, provides a convenient measure of stability. $T_m$ measurements are typically carried out in a saline solution at neutral pH with target and antisense

oligonucleotide concentrations at between about 1.0-2.0 $\mu$M. Typical conditions are as follows: 150 mM NaCl and 10 mM $MgCl_2$ in a 10 mM sodium phosphate buffer (pH 7.0) or in a 10 mM Tris-HCl buffer (pH 7.0); or like conditions. Data for melting curves are accumulated by heating a sample of the antisense oligonucleotide/target polynucleotide complex from room temperature to about 85-90°C. As the temperature of the sample increases, absorbance of 260 nm light is monitored at 1°C intervals, e.g. using a Cary (Australia) model 1E or a Hewlett-Packard (Palo Alto, CA) model HP 8459 UV/VIS spectrophotometer and model HP 89100A temperature controller, or like instruments. Such techniques provide a convenient means for measuring and comparing the binding strengths of antisense oligonucleotides of different lengths and compositions.

Another aspect of this invention is a pharmaceutical composition useful in inhibiting the synthesis of extracellular matrix proteins, comprising a pharmaceutically-acceptable excipient and an antisense oligonucleotide specific for nuclear proto-oncogene that is present in an amount sufficient to inhibit the synthesis of extracellular matrix proteins when administered to a subject in need thereof. Thus, the antisense oligonucleotides of the invention are employed as one or more components of such a pharmaceutical composition. Components of pharmaceutical compositions of the invention depend on several factors, including the nature of the disease or condition being treated, the location of disease lesions, the mode of drug delivery and/or administration contemplated, the latter of which can include *in vivo* administration by way of a catheter into a target lesion or organ, topical application, intranasal administration, administration by implanted or transdermal sustained release systems, and the like.

Pharmaceutical compositions of the invention include a pharmaceutical carrier that may contain a variety of components that provide a variety of functions, including regulation of drug concentration, regulation of solubility, chemical stabilization, regulation of viscosity, absorption enhancement, regulation of pH, and the like. For example, in water soluble formulations the pharmaceutical composition preferably includes a buffer such as a phosphate buffer, or other organic acid salt, preferably at a pH of between about 7 and 8. For formulations containing weakly soluble antisense compounds, microemulsions

may be employed, for example by using a nonionic surfactant such as Tween 80 in an amount of 0.04-0.05% (w/v), to increase solubility. Other components may include antioxidants, such as ascorbic acid, hydrophilic polymers, such as, monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, dextrins, chelating agents, such as EDTA, and like components well known to those in the pharmaceutical sciences, e.g. *Remington's Pharmaceutical Science*, latest edition (Mack Publishing Company, Easton, PA).

Sustained release systems suitable for use with the pharmaceutical compositions of the invention include semi-permeable polymer matrices in the form of films, microcapsules, or the like, comprising polylactides, copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, poly(2-hydroxyethyl methacrylate), and like materials, e.g. Rosenberg *et al*, International application PCT/US92/05305. Sustained release systems also include liposomally entrapped antisense compounds, e.g. as described in Liposome Technology, Vol. II, *Incorporation of Drugs, Proteins and Genetic Material* (CRC Press).

An effective amount of c-*myc* antisense oligonucleotide for particular applications depends on several factors, including the chemical nature of the antisense oligonucleotide, the disorder being treated, the method of administration, and the like. Preferably, an effective amount will provide a concentration of c-*myc* antisense oligonucleotide of between about 1 to 100 $\mu$M at the target polynucleotide; and more preferably, an effective amount will provide a concentration of antisense oligonucleotide of between about 1 to 10 $\mu$M at the target polynucleotide.

For vascular disorders, such as restenosis, antisense oligonucleotides specific for nuclear proto-oncogenes are preferably administered locally to the traumatized artery by way of a catheter. Preferably, the antisense oligonucleotides delivered to restenosis lesion are specific for c-*myc* and/or c-*myb*; more preferably, a combination of antisense oligonucleotides specific for both c-*myc* and c-*myb* is employed to reduce extracellular matrix protein synthesis associated with restenosis. Typically, restenosis occurs after a procedure such as percutaneous transluminal coronary angioplasty (PTCA) which causes a local disruption or injury to an arterial wall. In response to such injury, an occlusive lesion

frequently results from the following phenomena: inflammation of the of the artery at the injury site, thrombosis, the accumulation of smooth muscle cells by migration and proliferation, and smooth muscle cell and fibroblast synthesis of extracellular matrix.

For vascular graft disorders, oligonucleotides are preferably delivered to the connective tissue surrounding the graft to prevent graft failure. Generally, failure of a graft occurs when the loss of patency reaches 50 percent or more. It will be recognized that oligonucleotide delivery, as described by the techniques herein, can be targeted to arteriovenous grafts such as, but not limited to, prosthetic fistulae (including PTFE (Gortex or Impra), poly-(ether-urethane), dacron, human umbilical cord, genetically engineered veins and immunosuppressed bovine or porcine vessel), arteriovenous shunts, native vein fistulae, (including radial artery-cephalic vein (Brescia-Cimino), ulnar or radial artery-basilic vein, brachial artery-cephalic or brachial vein and femoral artery-saphenous grafts) and venous grafts such as, but not limited to, vein grafts using immunosuppressed saphenous vein allografts, immunosuppressed umbilical vein allografts, genetically engineered veins, immunosupressed xenografts, and venous autografts and any other veins that are compatible with humans, i.e., grafting tissue and materials capable of grafting to human tissue.

For perivascular administration of oligonucleotides to prevent vascular graft failure, particularly arteriovenous graft failure, oligonucleotides of the invention are generally injected in the perivascular tissue surrounding the site of intended application. In instances were continuous infusion of oligonucleotide is indicated, such as in patients with a known history of severe and rapid loss of arteriovenous graft patency, other types of delivery systems can be used such as subcutaneous infusion pumps, gels and biodegradable matrices molded onto the graft and preferably mixed with human serum albumin at a 1:10 to 1:100 ratio of oligonucleotide to protein, gels (such as pluronic, Hydron, or ethylene-vinyl acetate gels), gels (as described by Edelman *et al, Circulation Research* 76:176-182 (1995) and Edelman *et al., Proc. Natl. Aca. Sci.* 87:3773-3777 (1990) the methods of which are incorporated by reference), indwelling catheter systems (such as Broviac catheters or completely indwelling catheter reservoirs systems

such as those described by Poletti *et al.*, *J. Neurosurg.* 55: 581-584, the methods of which are incorporated by reference), impregnated PTFE tubing, other impregnated biocompatable materials or other drugs delivery compositions and systems described herein. It will be recognized that a reservoir can be made to release the oligonucleotide in close proximity to the site, in order to extend the duration of administration of the oligonucleotide. The reservoir can simply be made by injecting oligonucleotide into perivascular tissue during or after surgery, but before closure of the wound, or by percutaneous injection after palpating the arteriovenous graft, as in the case of an arteriovenous fistula for example, or by guiding the injection with methods known in the art, *e.g.* sonograms. Injections can be repeated for extended treatments, e.g., in hemodialysis acess sites. It is not necessary nor desirable to remove the adventitia from the vessel, since the perivascular tissue can act as a depot or reservoir of oligonucleotide that has a relatively slow rate of diffusion. Oligonucleotide in a reservoir is also more slowly carried away by the circulation system than when administered by intraluminal catheter. For percutaneous or perivascular administration the administered dose is in the range of .1 mg to 100 mg per vessel, preferably .25 mg to 50 mg and more preferably .5 mg to 10 mg per single administration.

In addition to the perivascular tissue administration of oligonucleotides to prevent graft failure, other anatomical structures such as the endothelial layer, media, and adventitia can be used as reservoir sites, particularly when the oligonucleotide is not applied from the lumen of the vessel. Such naturally occurring reservoir sites, including the perivascular tissue, are particularly useful for treating arteriovenous grafts, and more particularly arteriovenous shunts, where the media or elastic lamina is intact and not dissected, despite surgical injury. It is particularly advantageous to use sites near the vein portion of an arteriovenous graft, and more particularly the vein portion of an arteriovenous fistulae or shunt. The vein portion of an arteriovenous fistula forms the venous part the fistula, as exemplified in FIG. 1 with an arrow. It will be recognized that non-naturally occurring reservoirs can also supply a failing graft with oligonucleotide as shown with gray area in FIG. 1, examples of which are discussed herein. When non-naturally occurring reservoirs are used it is preferred

that they are either biodegradable or refillable. Refillable reservoirs are preferably of two types 1) a refillable reservoir secured upstream of the venous portion of the arteriovenous graft and in, or along, the PTFE tubing or other biocompatable material used to form an arteriovenous graft and 2) a reservoir secured in the perivascular tissue surrounding the arteriovenous graft, preferably within 5 cm of venous portion of the arteriovenous graft and more preferably within 2 cm, and refillable with a needle by percutaneous injection. Such non-naturally occurring perivascular reservoirs can be implanted during surgery to construct the arteriovenous graft. Refillable perivascular reservoirs can be made of materials used in breast implants and are preferably used in conjunction with a semipermeable membrane that permits the diffusion of oligonucleotide into the perivascular tissue.

For *ex vivo* application of oligonucleotides to prevent failure of vascular grafts made with veins, particularly for bypass procedures, the veins are contacted *ex vivo* with an oligonucleotide complementary to nuclear proto-oncogene mRNA. Generally veins are incubated for at least 10 minutes at temperatures between 20 and 37 °C in a sterile and physiologically acceptable buffer. Shorter times are permissible at particularly high concentrations. Longer incubation times between 30 and 90 minutes are also suitable and compatible with the time lag between vein removal and grafting experienced in such procedures. Lower incubation temperatures, such as 4 °C, may also be used, especially when the vein must be transported from a remote donor site, in conjunction with generally higher concentrations of oligonucleotide. Generally concentrations will vary from 5 to 500 $\mu$M depending on the length of incubation, type of oligonucleotide and temperature. Preferably, incubations times will be 30 to 60 minutes at temperatures ranging from 22 to 37 °C. Preferably oligonucleotide concentration will range from 10 to 100 $\mu$M and more preferably from 25 to 100 $\mu$M. It will be recognized that many types of buffers can be used so long as they physiologically acceptable, such as 0.9% saline (NaCl) or phosphate buffer saline, and other buffers and salts described herein. It will be recognized that many types of veins may be used with the invention including veins from non-humans, veins from genetically engineered mammals, especially pigs, and many human veins to from

allografts and autografts alike (such as saphenous vein, cephalic vein, basilic vein, brachial vein, and femoral vein).

For intravascular application (e.g. to prevent restenosis after angioplasty), antisense oligonucleotides are preferably administered in the vicinity of the lesion via catheter from inside the lumen, or through the adventitia (i.e. the most outer layer of the vessel wall) with materials aiding a slow release of antisense compound, e.g. a pluronic gel system as described by Simons *et al*, *Nature*, 359:67-70 (1992) or porous balloon or iontophoretic balloon as described by Fernandez-Ortiz, A., *et al.*, *Circulation*, 89:1518-1522 (1994), the methods of which are incorporated by reference herein. Other slow release techniques for local delivery include coated stents with antisense compound, e.g. using a binder or gel, described in Wilensky *et al*, *Trends in Cardiovascular Med.*, 3: 163-170 (1993). A dose delivered at the target lesion is in the range of from 1 $\mu$g to 10 mg for each or both of the antisense compounds employed in the pharmaceutical composition. Preferably the dose range is between 1 $\mu$g and 1 mg; and more preferably, the dose range is between 1 $\mu$g and 10 $\mu$g. Preferably, the delivery time is in the range of about 30 seconds to 60 minutes, and more preferably, in the range of about 30 seconds to about 1-2 minutes, e.g. Zalewski *et al*, pages 79-87 in Goldberg, editor, *Coronary Angioplasty* (Davis, Philadelphia, 1988).

For catheter administration, the administered dose is in the range of .1 mg to 10 mg per artery, preferably .25 mg to 4 mg per artery and more preferably .5 mg to 2 mg per artery. Administered dose is the amount drug applied to the tissue, where as "dose delivered at the target lesion" is in the amount of drug in the tissue after delivery to the tissue.

The techniques of coronary angioplasty are well known to those skilled in the art and are described in detail in such comprehensive treatises as Clark, Coronary Angioplasty (Liss, New York, 1987), Vlietstra *et al* (cited above), and the like.

For excessive scar formation in the skin, e.g. postsurgical scars, keloid disease, or the like, topical or intradermal application is preferred to inhibit

extracellular matrix protein synthesis, particularly collagen synthesis, by skin fibroblasts.

For topical application, the administered dose (amount of drug applied to tissue) will generally range from .01 $\mu$g to 10 mg of drug per $cm^2$, preferably from .2 $\mu$g to 7 mg of drug per $cm^2$, and more preferably from .5 to 4 mg of drug per $cm^2$. Administered dose for use with post-surgical dressing is preferably from .2 mg to 4 mg of drug per $cm^2$. The administered dose for use with topical creams for skin abrasions, skin lesions, acne and post-surgical treatments is preferably from .05 mg to 3 mg of drug per $cm^2$.

For urologic disorders resulting in urethral, ureteral, or bladder scarring or narrowing, local administration via catheter or implants coated with antisense oligonucleotide is preferred.

For excessive scarring of internal organs containing smooth muscle cells and fibroblasts, such as gastrointestinal organs, biliary ducts, lungs, and liver, administration of antisense compounds can be systemic or local. Local administration includes direct injection of antisense oligonucleotides into the target organ, delivery via implanted gels or polymers, or slow release from coated stents or other prosthetic materials.

For each of the disorders described above, criteria for selecting patients for treatment and means for assessing therapeutic endpoints are well known in the art concerning the specific disorders. For example, in regard to vascular restenosis factors associated with susceptibility to the condition include male gender, dilation for stenosis of bypass grafts, Canadian Heart class 3 or 4 at baseline, and history of myocardial infarction, Holmes *et al*, Chapter 12 in Vlietstra *et al*, editors, *PTCA* (Davis Company, Philadelphia, 1987). Efficacy of treatment is assessed in the same manner as in any PTCA technique. Usually, results are estimated visually, but other techniques are available. Assessment of the hemodialysis access site failure includes color Doppler ultrasound, contrast angiograph and ultrasound (*e.g.*, Dousset *et al*. *Radiology* 181:89 (1991), Nonnast-Daniel *et al*, *Lancet* 339 (8786):143 (1992), and Davidson *et al*, *Kidney International* 40: 91-95 (1991) methods which are incorporated by reference, and physical findings (such as high-pitched bruit over access site, or water hammer pulse). Assessment of the

severity of a stenosis, or restenosis (for determining a candidate for PTCA or the results of PTCA) range from visual observation of the lesion to complex computer-based analysis of the dimensions of the artery in question, e.g. from tomagraphical data, flow in the distal vascular bed, densitometric characteristic of the stenosis, or regional wall motion changes in the zone of the lesion, e.g. Bove, Chapter 9 in Vlietstra *et al* (cited above).

**EXAMPLE 1.** REMODELING OF ARTERIALIZED PORCINE VEIN - AN *IN VIVO* MODEL FOR THE LOSS OF PATENCY OF ARTERIOVENOUS GRAFTS

In order to demonstrate the effectiveness of an oligonucleotide of the invention in reducing extracellular matrix protein production in the venous portion of an arteriovenous graft, an *in vivo* model was first developed in pigs. This model permits monitoring of the patency of an arteriovenous graft, including the venous portion of the graft.

In the porcine arterialized vein model, saphenous veins were excised and then anastomosed, end-to-end, to carotid arteries. After surgery was completed, the arterial blood flow was allowed to resume to normal arterial pressures (Violaris *et al.*, *Ann. Thorac. Surg.*, 53: 667-71 (1995) and Angelini *et al.*, *J. Thorac. Cardiovas. Surg*, 99:433-9 (1990) methods of which are herein incorporated by reference). The high arterial pressure in the end-to-end saphenous vein graft simulates exposure of a vein to high arterial pressure in an arteriovenous graft or venous graft. Vein portions of venous grafts (n=7) were removed eight days after graft construction and sectioned for histological analysis, unless otherwise indicated. Normal coronary veins, unexposed to arterial pressure and ungrafted, were used for comparison.

Normal veins, unexposed to arterial pressure and ungrafted, showed no thickened perivascular tissue, a perivascular space with a small quantity of connective tissue and loose adipose tissue, and a thin and unbroken media, as shown in FIG. 2a (Verhoeff stain). Vein portions of grafts exhibited an increase in perivascular tissue that circumscribes the media as a collar with a large amount of granulation tissue. This was shown by Verhoeff staining, as shown in FIG. 2b.

The same section also exhibits an intact and unbroken media lacking dissection. In some sections neointima was observed at 8 and 14 days after surgery.

Normal veins, unexposed to arterial pressure and ungrafted, showed a small amount of loose perivascular and media staining with Sirrius red, as shown in FIG. 3a. In contrast, vein portions of grafts exhibited an increase in collagen deposition in the perivascular tissue as early as eight days. Large amounts of dense perivascular collagen were stained with Sirrius red, a specific stain for fibrillar collagen (including type I and III), as shown in FIG. 3b. The media showed less staining than the perivascular tissue. In sections with neointima, collagen was clearly present.

Normal veins, unexposed to arterial pressure and ungrafted, showed no α-actin in perivascular the and adventitia layers using α-actin antibodies. Only smooth muscle cells in the media stained positive for α-actin, as shown in FIG. 4a. In contrast, vein portions of grafts exhibited an increase in α-actin synthesis in the media and in the adventitia. Large amounts of α-actin in the media were stained with antibodies specific for α-actin, as shown in FIG. 4b. The staining in the media indicates the presence of smooth muscle cells synthesizing α-actin in the media. The adventitia also showed α-actin staining, indicating myofibroblast synthesis of α-actin. The perivascular tissue also showed some α-actin staining as well.

Veins were also stained for desmin, which is expressed by vascular smooth muscle cells, using anti-desmin antibodies. A normal vein, unexposed to arterial pressure and ungrafted, showed desmin staining only in media, as shown in FIG. 4c. Similarly, the vein portions of the venous grafts exhibited desmin staining of smooth muscle cell only in the media. Large numbers of smooth muscle cells in the media stained positive with an antibody for desmin, data not shown. No desmin staining was observed in the perivascular tissue or adventitia, indicating two populations of α-actin synthesizing cells: one population located in the media (smooth muscle cells; Desmin positive, α-actin positive) and qne population located in the adventitia (fibroblasts and myofibroblasts; Desmin negative, α-actin positive).

Vein portions of grafts exhibited an increase in cell proliferation in the media and perivascular tissue. Large numbers of proliferating cells in the media and perivascular tissue were stained with antibodies specific for proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, as shown in FIG. 5a. For comparison, a similar section was stained with Verhoff stain, FIG. 5b, and Sirrius red stain, FIG. 5c. In this section, early neointima formation can be observed in FIG. 5b as a thin dark layer a few cells in from the lumen of the grafted vein. Normally the neointima is absent from the vein. The greater staining for collagen in the perivascular tissue as compared to the media is more apparent at higher magnification, as shown in FIG. 5c. PCNA staining was also quantitated: 30% of the cells in grafted veins stained positive for PCNA, while less than one percent of the cells in non-grafted veins stained positive for PCNA.

These results confirm a relatively early increase of extracellular matrix production by fibroblasts, myofibroblasts and smooth muscle cells in the connective tissue and vessel wall of grafted veins responding to high arterial pressures. The increase in tissue thickness and cell proliferation around the vein, including the connective tissue in the perivascular space (scar formation), may prevent a favorable dilation of the vein in response to arterial blood pressure.

Inhibition of extracellular matrix production in vascular cells (e.g. smooth muscle cells and fibroblasts), *e.g.* collagen synthesis, is also specifically shown in Examples 6, 9, and 13.

**EXAMPLE 2.** HUMAN ARTERIALIZED VEIN - A MODEL FOR THE LOSS OF PATENCY OF ARTERIOVENOUS GRAFTS

In order to demonstrate the increase in extracellular matrix production as a consequence of vein arterialization in humans, human veins arterialized from by-pass surgeries were studied. The results from humans are striking, because essentially no cell proliferation was detected, however, marked collagen staining was present in the vessel wall.

Human saphenous veins that served as an aortocoronary bypass graft removed were removed from the patient approximately two years after an occlusion was diagnosed. The arterial blood flow exposes the vein to normal

arterial pressures. Vein portions of the aortocoronary grafts were sectioned for histological analysis.

Human vein portions of aortocoronary grafts exhibited an increase in perivascular tissue and neointimal thickness. This was shown by Verhoeff staining, as shown in FIG. 6a. Note the thickened neointima replaced the media and loss of patency of the vein. This particular vein is partially occluded due the loss of internal diameter of the vein. In contrast, normal human saphenous vein, unexposed to arterial pressure and ungrafted, showed no thickened perivascular tissue, and a thin, uninjured and undissected media, data not shown.

Human vein portions of aortocoronary grafts also exhibited a large amount of collagen in the perivascular tissue and media. Large amounts of collagen stained positive with Sirrius red, a specific stain for fibrillar collagen (including type I and III), in the perivascular tissue and media, as shown in FIG. 6b.

Human vein portions of grafts exhibited small amounts of in $\alpha$-actin in the neointima and no $\alpha$-actin in the perivascular tissue, except for positive staining in vasa vasorum, with antibodies specific for $\alpha$-actin, as shown in FIG. 6c. The lack of staining in the media and perivascular tissue indicates that few cells were producing $\alpha$-actin. Instead of staining positive for $\alpha$-actin, these sections as shown in FIG. 6b stained more heavily for collagen, indicating that scar tissue had filled in both the perivascular tissue and the vessel wall.

Human vein portions of arteriovenous grafts exhibited no cell proliferation in the media and perivascular tissue. No cells in the media and perivascular tissue were stained with antibodies specific for proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, as shown in FIG. 6d. Even at x25 magnification few positive staining cells were found. PCNA staining was also quantitated and less than one percent of the cells in grafted human veins stained positive for PCNA.

**EXAMPLE 3.** HUMAN AND PORCINE BLOOD VESSEL ORGAN CULTURES - A MODEL FOR THE EFFECTIVENESS OF NON-LUMINAL DELIVERY OF OLIGONUCLEOTIDE AND INHIBITION OF VESSEL RESPONSE TO INJURY

In order to demonstrate the effectiveness of an oligonucleotide of the invention in reducing extracellular matrix protein production and vascular compromise after arteriovenous shunt or bypass procedures an organ culture model was first developed. This model permits monitoring of oligonucleotide uptake, stimulation of vascular cells by platelet derived growth factor B-chain (referred to herein as "PDGF") and inhibition of PDGF stimulation of vascular cells by oligonucleotides of the invention.

For the porcine blood vessel organ culture, internal mammary or carotid arteries were excised (n = 3) and sectioned into rings approximately 5-15 mm in thickness. The rings were incubated under organ culture conditions for variable times as indicated below. For the human blood vessel culture model, saphenous veins were excised from patients undergoing bypass surgery. After excision was completed, unused vein segments were sectioned into rings approximately 5-15mm in thickness.

To assess the affect of c-*myc* oligonucleotides on cell proliferation porcine vascular rings were incubated at 37 °C in serum-free media (DMEM (Dulbecco's Modified Eagle's Medium) with antibiotics and 1% Neutrodoma) for four days with or without 100 ng per ml PDGF, unless otherwise indicated. PDGF normally stimulates vascular cells, e.g., smooth muscle cells and fibroblasts, to proliferate and is known to be released by platelets after tissue injury to promote wound healing. PDGF induced cell proliferation was measured by PCNA staining as discussed herein. Even at four days, the rings maintained their morphology.

Porcine blood vessels in organ culture responded to PDGF stimulation. Porcine blood vessels in organ culture showed cells staining positive for PCNA in the adventitia and media, as shown in FIG. 7a. The brown nuclear staining represents PCNA stained cells, while the purple stained cells are stained with hematoxylin and eosin as a counter stain. The percentage of cells staining positive for PCNA was 15 $\pm$ 5 percent (n = 3). The control, blood vessels incubated only in serum free media without PDGF, showed no significant PCNA staining.

Less than one percent of the cells staining positive for PCNA, as shown in FIG. 7b.

Oligonucleotides of the invention inhibited PDGF-induced stimulation of porcine blood vessel organ cultures. The addition of oligonucleotide (SEQ ID No:1) at 16 $\mu$M inhibited PDGF induced cell proliferation, as shown in FIG. 7c. After four days of incubation with oligonucleotides PCNA staining was 2 $\pm$ .2 percent of the cells ($n = 3$; $p < 0.001$ versus PDGF alone).

To access oligonucleotide diffusion into the vessel wall of human veins, human saphaneous veins were incubated with oligonucleotide. Oligonucleotides of the invention rapidly diffused into human vein organ cultures. The addition of fluorescein end-labeled oligonucleotide (SEQ ID No:1) at 100 $\mu$M to the organ culture media produced rapid, time-dependent diffusion of labeled oligonucleotide into human veins at incubation times of 2 seconds (room temperature), 30 minutes (37°C), 1 hour (37°C) and 3 hours (37°C), as shown in FIG.s 8a - 8d, respectively. The labelled oligonucleotides diffused across both the luminal surface of the veins and perivascular surface of the veins. Oligonucleotides, over time, localized in both cells and cell nuclei. The control, human veins incubated without oligonucleotide, showed little autofluorescence, even at shutter exposure times 2-3 times longer than the shutter times for the oligonucleotide incubated veins.

This data is consistent with the oligonucleotides of the invention inhibiting PDGF-induced stimulation of vascular cells in the vessel wall. This data also demonstrates the effectiveness of oligonucleotide delivery from the perivascular tissue of blood vessels, instead of using luminal delivery of oligonucleotides. In particular, perivascular tissue oligonucleotide delivery can be used *ex vivo* to deliver oligonucleotides to excised veins or arteries used for venous grafts and other types of vascular grafts. In addition, the perivascular tissue can be treated with oligonucleotides of the invention *in vivo* allowing for oligonucleotide transfer to the vessel wall, in order to prevent vessel wall and perivascular tissue thickening.

**EXAMPLE 4.** TWO *IN VIVO* PORCINE MODELS DEMONSTRATE PERIVASCULAR OLIGONUCLEOTIDE DELIVERY

In order to demonstrate the effectiveness of perivascular delivery of oligonucleotides of the invention, two *in vivo* porcine models were tested. In the first model the porcine femoral vein was surgically exposed and oligonucleotide injected around it in the connective tissue of the perivascular tissue. In the second model, no surgery was performed, and the connective tissue around the porcine veins were percutaneously injected with oligonucleotide. These models permitted the monitoring of oligonucleotide uptake after administration by either 1) perivascular injection before, or after, construction of the arteriovenous graft, but before closure of the surgical site or 2) percutaneous injection to the perivascular tissue if repeated administration is required postsurgery. Fluorescein end-labeled oligonucleotides (SEQ ID No:1) were injected at 100 $\mu$M concentration (10 mg) in both models using a 22 gauge needle.

*In vivo* oligonucleotide uptake was rapid after direct, perivascular application of oligonucleotide to a surgically exposed vein. Fluorescence could be observed in the nuclei of the cells located in the vessel and perivascular tissue within thirty minutes (data not shown) and at two hours (FIG. 9a) indicating that oligonucleotides had diffused across the diffusion barriers of the vessel wall and perivascular tissue.

*In vivo* oligonucleotide uptake was also rapid after percutaneous injection. Fluorescence could be observed in the nuclei of the cells located in the vessel and perivascular tissue within thirty minutes (FIG. 9b) and three hours (FIG. 9c) indicating that oligonucleotides had diffused across the diffusion barriers of the vessel wall and perivascular tissue.

These results establish perivascular delivery as a way of administering oligonucleotides of the invention to grafts, in particular perivascular delivery of oligonucleotides to arteriovenous and venous grafts. Generally, oligonucleotides can be injected as shown here, or oligonucleotides can be delivered via other means that allow delivery of the oligonucleotide to the perivascular tissue, such as implantable reservoirs, time release gels or matrices, and oligonucleotide impregnated tissue, or devices, such as PTFE tubing, as discussed herein. These

types of perivascular delivery methods can be used to deliver oligonucleotides of the invention to prevent thickening of the perivascular tissue or vessel wall in arteriovenous grafts, especially the venous portion of hemodialysis access sites used by hemodialysis patients. The success of percutaneous injections also indicates the effectiveness of repeated delivery of oligonucleotides to an arteriovenous graft, in particular hemodialysis access sites used by hemodialysis patients. In addition to showing successful oligonucleotide delivery to the perivascular side of veins, luminal delivery to arteries was also shown in Examples 8, 18 and 19.

**EXAMPLE 5.** HEMODIALYSIS ACCESS SITE CLINICAL PROTOCOL

Patients requiring hemodialysis access sites for the first time or replacement of failed sites will be treated with the oligonucleotides of the invention. Either PTFE type fistulae or Brescia-Cimino fistulae will be treated.

Patients will receive oligonucleotides with the sequences of SEQ ID No. 1, 5, or 6. The oligonucleotides will contain phosphorothioate linkages or amidate linkages. Lyophilized oligonucleotides with phosphorothioate linkages are suspended in a buffer as described herein. Oligonucleotides with phosphorothioate or amidate linkages are synthesized according to procedures discussed herein and maintained under sterile conditions. Oligonucleotides with different sequences will be separately administered and patients are to receive only one sequence during the treatment.

Patients are administered 1-10 mg of oligonucleotide at the time of hemodialysis access site surgery. Oligonucleotides are injected into the perivascular tissue on the venous side of the arteriovenous shunt using a standard 22 gauge or smaller needle. The surgery is completed after injection. Patients are repeatedly administered 1-10 mg of oligonucleotides using percutaneous injections at weekly intervals for six months. Percutaneous injections in patients with difficult to palpitate veins will be guided using sonograms. Otherwise injections will be guided by palpation of the arteriovenous shunt area, in particular the vein portion of the shunt.

Patients are monitored for the functional patency of hemodialysis access site during the first year. Sonograms or angiograms of the site are taken at 1 week, and three, six, and twelve months. Flow rates will be determined using Doppler and ultrasound techniques, discussed herein.

The oligonucleotide used for administration is selected and synthesized as discussed herein and known in the art. The sequences and methods discussed in this example and other examples should not be construed as limiting the scope of the invention but as specific embodiments of the invention. Further oligonucleotides need only be consistent with the structure proposed herein. It is not necessary for oligonucleotides to operate by a particular mechanism of action proposed herein, so long as the oligonucleotides test favorably in *in vitro* and *in vivo* models used herein.

**EXAMPLE 6.** INHIBITION OF COLLAGEN SYNTHESIS IN HUMAN SMOOTH MUSCLES CELLS

In this example, explanted human smooth muscle cells committed to collagen synthesis were treated with c-*myc* or c-*myb* antisense oligonucleotide and compared with untreated and/or "sense" treated controls.

Human smooth muscles cells (SMCs) originated from the saphenous veins of patients undergoing routine bypass surgery. The cells were isolated by an explant method. The explants were placed into tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM/ml glutamine (CM-20). The cultures were maintained at 37°C in a humidified incubator with 5% $CO_2$. The cells exhibited typical morphological characteristics of vascular smooth muscle cells i.e., spindle shape and hill-and-valley pattern. The identification of vascular smooth muscle cells was further confirmed by *in situ* smooth muscle $\alpha$-actin staining. Cells were grown to confluence and subcultured every 7 days. Only 3rd or 4th passage human smooth muscle cells were used for experiments.

The experiments were carried out in subconfluent and post confluent human smooth muscle cells. Cells were plated at 10.000/cm$^2$ (subconfluent) or

25,000/cm$^2$ (post confluent) supplemented with CM-20. Three days after plating, fresh CM-20 containing ascorbic acid (50 $\mu$g/ml) was added. The following oligonucleotides were added (16$\mu$M) to the cultures in the various experiments: antisense (5'-AACGTTGAGG GGCAT)(SEQ ID No:1); sense oligomers (5'-ATGCCCCTCA ACGTT)(SEQ ID No:2); 4 bp mismatch oligomer (5'AACGTGGATT GGCAG)(SEQ ID No:3); scrambled oligomer (5'-GAACGGAGAC GGTTT)(SEQ ID No:4); or c-*myb* antisense (5'-TATGCTGTGC CGGGGTCTTC GGGC)(SEQ ID No:5).

Twenty four hours later, the culture medium was collected for Western blots and the cells were harvested for RNA analysis. A trypan blue exclusion assay was carried out with each experiment.

Collagen was measured as follows: Both pepsin treated (triple helical portion of collagen I) or non-pepsin treated samples were subjected to Western blot for collagen measurement. Briefly, the samples were incubated at 4°C overnight in pepsin (1 mg/ml) and acetic acid (0.5 mol). Equal volume aliquots of samples were then concentrated in a speed vacuum centrifuge, after which samples were dissolved in SDS gel loading buffer (50mM tris • HCl pH 6.8, 100mM dithiothreitol, 2% SDS, O.1% bromophenol blue and 10% glycerol) and boiled for 10 min. Following centrifugation at 11,000 g for 10 min., samples were electrophoresed through 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose filter. After incubation in blocking solution, the sample-containing filters were incubated with biotin labeled primary antibody against human Collagen I and III (Southern Biotech. SC.) for 1 hour at room temperature. The filters were washed and then incubated with streptavidine labeled with horseradish peroxidase. Following addition of the reaction substrate, the filters were exposed to X ray film. The experiments were repeated at least twice on separate occasions.

Human smooth muscle cells used in all experiments underwent 3-4 passages. The phenotype of these cells is considered to be irreversibly synthetic and is associated with various disease states. It has been demonstrated that abnormal collagen accumulation in the vessel wall was mainly produced by these

synthetic cells. Both subconfluent and postconfluent smooth muscle cells produced type I and type III collagens under serum stimulation.

In subconfluent smooth muscle cells, there was 80% reduction in collagen type I synthesis after c-*myc* antisense oligonucleotide treatment as compared with control (no oligonucleotide added) and c-*myc* sense oligonucleotide treated cells. In confluent smooth muscle cells, cell numbers counted in the Coulter counter were similar among control, antisense- and sense-treated cells. Viability of cells were 97%, 96% and 95% in the control, antisense- and sense-treated cells using trypan blue exclusion assay. Collagen type I was nondetectable in c-*myc* antisense-treated cells. Cells treated with c-*myc* sense oligomer showed no difference in the level of collagen type I protein as compared with control. Triple helical type I collagen was measured after digestion and the results showed the same trends with non-pepsin treated samples.

In subconfluent smooth muscle cells, there was 80% reduction in procollagen type I synthesis after c-*myc* antisense oligonucleotide (16 $\mu$M) treatment as compared with the control (no oligonucleotide added) and c-*myc* sense oligonucleotide treatment. In postconfluent smooth muscle cells, collagen type I was nondetectable in c-*myc* antisense treated cells. Cells treated with sense c-*myc* oligonucleotides and 4 bp mismatch oligonucleotides showed no reduction in the level of collagen type I protein as compared to the control (no oligonucleotide added). The reduction of collagen type I in both proliferating and postconfluent smooth muscle cells suggests that the inhibition of collagen synthesis is independent of inhibition of cell growth by antisense compounds. Different doses (4, 8, and 16 $\mu$M) of antisense oligonucleotide produced a dose dependent reduction of procollagen type I synthesis. Cell viability following different oligonucleotide treatments was comparable, as analyzed by a trypan blue exclusion assay. More than 90% cells remained viable in all groups.

In postconfluent human smooth muscle cells, the synthesis of collagen type I was abolished by c-*myb* antisense oligonucleotide at a concentration of 16 $\mu$M. In the control (no oligonucleotide) and cells treated with scrambled sequence oligonucleotides, the level of collagen type I was similar.

These results are also further confirmed in Examples 9, 13 and 17.

**EXAMPLE 7.** INHIBITION OF COLLAGEN SYNTHESIS IN HUMAN SKIN FIBROBLAST CELLS

In this example, explanted human skin fibroblast cells (human FBCs) were treated with c-*myc* or c-*myb* antisense oligonucleotide and compared with untreated and/or "sense" treated controls. Human FBCs were obtained from patients undergoing skin transplants and were isolated by an explant method. Explanted FBCs were cultured as described above for human smooth muscle cells and antisense and control oligonucleotides were applied in an identical manner. Human FBCs from 3rd to 10th passage cultures were used in the experiments. Collagen synthesis was measured as described in Example 6.

In confluent human FBC, c-*myc* antisense oligonucleotides (8 and 16 $\mu$M) reduced procollagen type I synthesis in a dose dependent manner. Cells treated with c-*myc* sense, 4 bp mismatch, or scrambled oligonucleotides showed similar levels of collagen type I protein as compared with control cells (no oligonucleotide added).

These results are also further confirmed in Example 13.

**EXAMPLE 8.** REDUCTION OF NEOINTIMA FORMATION IN PORCINE MODEL OF CORONARY DENUDATION

The effectiveness of c-*myc* antisense compounds to inhibit neointima formation was tested by administering c-*myc*-specific antisense (SEQ ID No:1) and placebo (SEQ ID No:21) oligonucleotide phosphorothioates to the site of coronary angioplasty in a standard porcine model using conventional protocols, e.g. see Karas *et al*, *J. Am. Coll. Card.*, 20:467-474 (1992); and Schwartz *et al*, *Circulation*, 82:2190-2200 (1990). Domestic crossbred pigs *(Sus scrofa)* were premedicated with oral aspirin (650 mg) prior to the study. General anesthesia consisted of intramuscular injection of ketamine (12 mg/kg) and xylazine (8 mg/kg). Additional doses of anesthesia were given intravenously throughout the experiment. After the right external carotid artery was surgically exposed, heparin (10,000 U) was administered to the pig intravenously, and nifedipine (10 mg) was given buccally. Using an 8 French SAL 1 guiding catheter (Medtronic Interventional Vascular, Inc., Danvers, MA) the coronary ostia were cannulated

under fluoroscopic guidance. Following intracoronary injection of nitroglycerin (100 μg), and prior to the delivery of the c-*myc* antisense and placebo, an oversized angioplasty balloon was used to injure the intimal and medial layers of the arterial walls by inflating at 6-10 atm, according to the size of the artery which varied from 2.0 to 3.5 mm, and holding for 30 seconds three times in succession. Immediately after the angioplasty balloon was removed, intramural injections (1 mg/vessel) to the coronary arteries were carried out using a separate porous balloon. It was predetermined that intramural drug delivery was safe when the injection pressure did not exceed 4 atm, the volume of perfusate was 2 ml, and the balloon-artery ratio was about 1.4 to 1. The c-*myc* antisense (13 replicates) or placebo (12 replicates) oligomers were injected under 4 atm of pressure and delivery was completed in an average of 27 seconds. The dose of oligomers was 1 mg per injured coronary artery. No adverse effects were associated with the delivery of the oligomers. One month after delivery, the animals were sacrificed and the maximal neointimal area (NA max), the neointimal thickness (NT max), and the residual lumen (RL) at the injury sites were determined by morphometry. The results (mean ±SEM) are shown in Table I below:

**TABLE I**

| Oligomer (%) | Replicates | NA max ($mm^2$) | NT max (mm) | RL |
|---|---|---|---|---|
| placebo ± 6 | 12 | O.80 ± O.17 | 0.48 ± 0.09 | 64 |
| antisense ± 5 | 13 | 0.24 ± 0.06 | 0.20 ± 0.04 | 81 |
| *p* <0.05 | | <0.01 | <0.01 | |

FIG. 10 is a photograph of a cross-section of an exemplary control (i.e., which received sense oligomer injection) coronary artery one month following

injury. A significant neointimal thickness is noted (arrows). FIG. 11 is a photograph of a crosssection of an exemplary antisense-treated coronary artery. A marked reduction of the neointima is noted. When maximal neointimal area was analyzed as a function of degree of injury (FIG. 12), regression lines representing the relationship between neointima and injury score (i.e., the severity of injury) showed a significant difference by slopes ($p<0.01$). As shown in FIG. 12, antisense oligomers significantly reduced neointimal formation, especially with more advanced injury.

**EXAMPLE 9.** INHIBITION OF EXTRACELLULAR MATRIX PRODUCTION IN HUMAN SMOOTH MUSCLE CELLS BY *C-MYC* ANTISENSE OLIGONUCLEOTIDE TREATMENT

This example further demonstrates the effectiveness of *c-myc* antisense oligonucleotide treatment to inhibit the synthesis of extracellular martrix protein by human smooth muscle cells, in particular procollagen I and III. This example also further confirms the findings of Example 6. Antisense treatment greatly reduced extracellular procollagen levels in stimulated human smooth muscle cells (treated with 20% FBS).

*A. Measurement of procollagen I and procollagen III by Western blot.*

To further determine that *c-myc* antisense oligonucleotides inhibited the synthesis of extracellular matrix proteins, procollagen I & III was measured from human smooth muscle cells.

Both intracellular and extracellular procollagen (both type I & III) were measured using Western blots. Resting human smooth muscle cells were maintained in medium DMEM (Dulbecco's Modified Eagle's Medium) with .5% FBS (fetal bovine serum). Human smooth muscle cells were stimulated with or without oligonucleotides in medium containing 20% FBS and ascorbic acid (50 $\mu$g/ml). FBS is known to stimulate procollagen synthesis in human smooth muscle cells. At 20 hours after the addition of oligonucleotides cells were washed threes times with phosphate-buffered saline (PBS) and serum-free medium containing 0.1% BSA, ascorbic acid and oligonucleotides was added to the culture for an additional 4 hours. The conditioned medium (medium exposed to cells and oligonucleotides) was collected and proteinase inhibitors including

phenylmethylsulfonyl fluoride (PMSF, 0.5 mM), pepstatin (1 μg/ml) and leupeptin (1 μg/ml) were added. The corresponding cell layer was washed three times with cold PBS and lysed in SDS-PAGE sample buffer containing 62.5 mM Tris-HCl, pH 6.8. 20% glycerol, 2% SDS, 5% β-mercaptoethanol for 30 minutes at 4°C. Cell lysates were then boiled for 5 minutes to inactivate proteinases and the supernatants were collected after centrifugation at 10,000 rpm for 10 minutes at 4°C. In selected experiments, cells were continuously incubated with oligonucleotides in 20% FBS-DMEM for 24 hours and samples of culture medium were then subjected to the overnight pepsin digestion (0.1 mg/ml) in 0.5 M acetic acid at 4°C. Individual samples were concentrated 10 fold by centrifugation through a Microcon-30 column (Amicon, Beverly, MA) and stored at -20°C. Aliquots were electrophoresed on 65 (w/v) polyacrylamide-SDS gels with 4% stacking regions. The fractionated proteins were then electrotransferred onto PolyScreen PVDF membranes (DuPont NEN, MA) in a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.01% SDS, and 20% methanol. The blots were blocked in 5% non-fat milk and incubated with antibodies against type I collagen (biotin-labeled polycolonal antibody, Southern Biotechnology Associates, Inc. AL) and against type III collagen (Gibco BRL, Gaithersburg, MD) in 2.5% BSA. The membranes were washed 3 times with PBST (PBS containing 0.1% Tween 20) and incubated with biotinylated secondary antibodies (for type III). The blots were washed and then incubated with streptavidin-peroxidase conjugate (Boehringer Mannheim, Germany) for 30 minutes. After washing, the blots were incubated with Renaissance chemiluminescence reagent (DuPont NEN, MA) for 10 seconds and exposed to Kodak X-Omat film for 30 seconds to 2 minutes. Resulting fluographs were analyzed by laser densitometry (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

*c-myc* antisense oligonucleotides corresponding to SEQ ID No:1 were used in all examples, unless otherwise indicated.

*B. Inhibition of the Extracellular Procollagen I and Procollagen III Secretion By c-myc Antisense Oligonucleotides*

Procollagen levels were determined in post-confluent i.e., density-arrested smooth muscle cells in order to minimize the influence of various growth rates among experimental subgroups. A marked reduction (90%) of extracellular procollagen I and extracellular procollagen III in the conditioned medium was observed following *c-myc* antisense oligonucleotides treatment as shown by Western blot (FIG. 13). This effect was concentration-dependent in a range of 4 - 16 $\mu$M and it occurred as early as 3 hours following incubation of smooth muscle cells with *c-myc* antisense oligonucleotides (data not shown).

The reduction in extracellular procollagen I after antisense oligonucleotides was unaffected by the addition of $\beta$-aminopropionitrile excluding the possibility that antisense promoted cross-linking of collagen (data not shown).

*C. c-myc Antisense Phosphorothioate Oligonucleotides To Exons 1, 2 And 3 Are Effective Inhibitors Of The Synthesis Of Extracellular Matrix Proteins.*

To further confirm a sequence-specific action of *c-myc* antisense phosphorothioate oligonucleotides, smooth muscle cells were treated with several control sequences including sense, 4 bp-mismatched and scrambled oligonucleotides (phosphorothioate oligonucleotides are used herein unless otherwise indicated). Their effects on procollagen I levels in the conditioned medium were compared with those after three antisense sequences directed against different regions of *c-myc* mRNA. As shown in FIG. 14, a consistent reduction in extracellular procollagen I was observed after treatment with different antisense oligonucleotides, whereas control sequences were devoid of such effect.

TABLE III

| SEQ. ID. NO. | OLIGONUCLEOTIDE SEQUENCE (5'→3') | HUMAN *c-myc* NUCLEOTIDE LOCATION; OLIGONUCLEOTIDE TYPE | |
|---|---|---|---|
| 1 | AACGTTGAGG GGCAT | 559-573; | Antisense |
| 2 | ATGCCCCTCA ACGTT | 559-573; | sense |
| 3 | AACGTGGATT GGCAG | 559-573; | four base mismatch |
| 4 | GAACGGAGAC GGTTT | Scrambled | |
| 5 | TATGCTGTGC CGGGGTCTTC GGGC | *c-myb*; | Antisense |
| 6 | GGGAGAGTCG CGTCCTTGCT | 400-419; | Antisense |
| 7 | TCATGGAGCA CCAGGGGCTC | 1264-2383; | Antisense |

*D. Immunoprecipitation of Extracellular Fibronectin and Nuclear c-myc* Protein

To determine the effects of oligonucleotides sequences on protein synthesis of extracellular fibronectin and *c-myc* protein, post-confluent smooth muscle cells were incubated with or without oligonucleotides in methionine-free medium for 4 hours. Afterwards, [$^{35}$S]methionine (25 $\mu$Cl/ml, DuPont NEN, MA) was added for 16 hours and the cell number was counted in a Coulter counter. Extracellular fibronectin in the conditioned medium and *c-myc* protein in cell nuclear extracts were measured by immunoprecipitation following metabolic labeling.

The conditioned medium was collected after the addition of proteinase inhibitors and aliquots containing equal counters were incubated with antibodies against fibronectin (Becton Dickinson, Bedford, MA) for 4 hours followed by Staphylococcus protein A acrylamide beads for an additional 2 hours. After centrifugation, pellets were washed three times, boiled in SDS-PAGE sample buffer for 5 minutes and electrophoresed on a 6% polyacrylamide-SDS gel. The labeled cell layers were washed three times with cold PBS after labeling and lysed in 0.1% NP-40, 10mM Tris (pH 7.9), 10mM $MgCl_2$, 15 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 $\mu$g/ml leupeptin and 1 $\mu$g/ml pepstatin.

In parallel to the extracellular fibronectin assays, cell nuclei were extracted through centrifugation by procedures unknown in the art. The protein concentration in nuclear extracts was measured and aliquots containing 500 $\mu$g of

protein were incubated with anti-*c-myc* antibody, pan-*myc*, (gift from G.I. Evan), for 12 hours and then with Staphylococcus protein A acrylamide beads for an additional 90 minutes. After washing, the beads were collected and boiled in SDS-PAGE sample buffer and the soluble fraction was electrophoresed on SDS-PAGE. The gel was dried and exposed to Kodak-Omat film at -70°C for 5-7 days.

*E. Levels of Extracellular Fibronectin Were Unchanged By c-myc Antisense Oligonucleotides, While c-myc Protein Levels Decreased*

In contrast to a significant reduction in extracellular procollagens, the levels of extracellular fibronectin in the conditioned medium were not significantly affected (FIG. 13) pointing to selective effects of antisense oligonucleotides. The incubation of post-confluent smooth muscle cells with *c-myc* antisense oligonucleotides (16 $\mu$M) resulted in downregulation of *c-myc* protein in nuclear extracts (FIG. 15).

**EXAMPLE 10.** *C-MYC* ANTISENSE OLIGONUCLEOTIDES DO NOT ALTER HUMAN SMOOTH MUSCLE CELL OVERALL METABOLISM

To further ensure that oligonucleotides did not produce other changes in smooth muscle cell function, assays of cell membrane integrity, cell metabolism and induction of genes related to collagen synthesis or degradation pathways were conducted.

Human smooth muscle cells were assayed for cell membrane integrity using trypan blue. More than 95% of cells remained viable following treatment with various oligonucleotides (16 $\mu$M) as assessed by trypan blue exclusion.

To determine total protein synthesis aliquots of the conditioned media were precipitated in cold 10% trichloroacetic acid (TCA) for 30 minutes at 4°C and pellets were washed twice in 5% TCA. The pellets were then dissolved in Solvable™ (DuPont NEN, MA) and the incorporated radioactivity was measured in a scintillation counter (Pharmacia LKB Biotechnology Inc. Piscataway, NJ). The overall protein synthesis, as measured by [$^{35}$S]methionine incorporation as discussed herein, was comparable in smooth muscle cells incubated with or without antisense (SEQ ID No:1) oligonucleotides (FIG. 16). Note that cells

stimulated with 20% FBS showed increased protein synthesis compared to unstimulated cells treated with only .5% FBS. *c-myc* antisense oligonucleotide treatment did not reduce protein synthesis compared to sense, mismatch and no oligonucleotide controls.

In addition, the level of procollagen I in the conditioned medium was restored to control levels 24 hours after the removal of *c-myc* antisense oligonucleotides (FIG. 17). These results indicated that antisense (SEQ ID No:1) oligonucleotides produced a selective but reversible reduction of extracellular procollagens preserving normal metabolic activity of vascular smooth muscle cells.

Because antisense oligonucleotides may induce expression of non-targeted genes, the collagenolytic activity and interferon-$\gamma$ levels following treatment with *c-myc* antisense oligonucleotides was determined to exclude these non-specific effects.

Post-confluent smooth muscle cells were incubated with serum-free medium with or without oligonucleotides. The aliquots of medium were collected 24 hours thereafter. Matrix metalloproteinase activities were determined after selective destruction of tissue inhibitor of metalloproteinase by dithiothritol and iodoacetamide. Latent collagenases produced by human smooth muscle cells were activated with aminophenylmercuric acetate, and then incubated with type 1 collagen or gelatin and cleavage products assessed by SDS-PAGE (the methods of D.D. Dean, *et al.*, J. Clin. Invest., 84:678-685 (1989) and P.E. Desrochers, *et al.*, J. Biol. Chem 267:5005-5012 (1992) are incorporated by reference herein). Metalloproteinases were characterized by SDS-substrate gel electrophoresis (i.e., zymography) under nondenaturing conditions using either $\beta$-casein or gelatin as substrates. Comparable collagenolytic activity against type I collagen and gelatin were exhibited in conditioned medium from control, sense- and antisense- (SEQ ID No:1) treated smooth muscle cells (data not shown), suggesting that *c-myc* antisense oligonucleotides did not increase endogenous collagenolytic activity.

To determine whether *c-myc* interferon-$\gamma$, a potent inhibitor of type I collagen synthesis, was inadvertently induced, interferon-$\gamma$ was assayed in the culture medium and human smooth muscle cells. Interferon-$\gamma$ was undetectable in

the conditioned culture media and cell lysates after incubation with *c-myc* antisense (SEQ ID No:1) oligonucleotides using ELISA method.

**EXAMPLE 11.** NO EFFECT OF *C-MYC* ANTISENSE OLIGONUCLEOTIDES ON PROCOLLAGEN $A_1(I)$, $A_2(I)$ AND $A_1(III)$ MRNAS IN HUMAN SMOOTH MUSCLE CELLS

This example demonstrates that *c-myc* antisense oligonucleotide treatment does not decrease the mRNA of procollagens in human smooth muscle cells.

To measure mRNA from procollagen post-confluent smooth muscle cells were incubated with or without oligonucleotides for 24 hours, washed three times with cold PBS and lysed in 4 M guanidinium isothiocyanate. Total RNA was isolated by phenol-chloroform extraction followed by isopropanol precipitation. The total RNA was quantitated by spectrophotometric absorbance at 260 nm. Equal amounts of RNA samples (10 $\mu$g) were denatured and separated on 0.8% agarose/formaldehyde gels and blotted onto nitrocellulose membranes. The RNA was crosslinked to nitrocellulose membranes using a UV crosslinker (Stratagene, CA). The blots were hybridized at 42°C for 24 hours with human procollagen $\alpha_1(I)$ (ATCC) and human procollagen $\alpha_2(I)$ (gift from H. Kuivaniemi) cDNA probes. The probe for 7S ribosomal RNA were used as control The probes were labeled with $^{32}$P-CTP by nick translation to a specific activity of greater than $10^8$ cpm/$\mu$g DNA. The blots were sequentially washed twice in 2x, 1x, 0.5x and 0.1x SSC-0.1% SDS at 42°C for 15 min. Each wash was repeated twice. Blots were exposed to Kodak-Omat film at -70°C with an intensifying screen for 6 hours to 3 days.

Downregulation of *c-myc* protein after antisense treatment raises the possibility of transcriptional regulation of collagen expression. Accordingly, procollagen $\alpha_1(I)$ and procollagen $\alpha_2(I)$ mRNA levels were analyzed in smooth muscle cells after their incubation with or without oligonucleotides using Northern blot analysis. As shown in FIG. 18, antisense oligonucleotides did not affect procollagen $\alpha_1(I)$ and procollagen $\alpha_2(I)$ mRNA levels. Likewise, similar levels of procollagen $\alpha 1(III)$ mRNA were seen with or without *c-myc* antisense

oligonucleotides (data not shown). 7S RNA remained unchanged in all three experimental conditions.

**EXAMPLE 12.** INTRACELLULAR ACCUMULATION OF PROCOLLAGEN IN HUMAN SMOOTH MUSCLE CELLS TREATED WITH *C-MYC* ANTISENSE OLIGONUCLEOTIDES

This example demonstrates that *c-myc* antisense oligonucleotide treatment increases procollagen inside human smooth muscle cells. These results are consistent with the decrease in extracellular matrix procollagen synthesis in the presence of *c-myc* antisense oligonucleotides.

*A. Intracellular Procollagen Levels Are Increased by c-myc Antisense Treatment.*

To examine mechanisms involved in the reduction of extracellular procollagen by *c-myc* antisense oligonucleotides, the secreted and intracellular procollagens were determined following 4 hours of [$^{14}$C]proline labeling.

To measure intracellular procollagen after *c-myc* antisense oligonucleotide treatments, cells were incubated with or without oligonucleotides for 20 hours in 20% FBS-DMEM. Serum-free medium containing ascorbic acid (50 $\mu$g/ml), [$^{14}$C]proline (2.5 $\mu$CI/ml, DuPont NEN, MA) and oligonucleotides were added to the cells for the additional 4 hours. The culture medium was collected and proteinase inhibitors were added. Aliquots of media were centrifuged through Microcon-30 column in order to remove unincorporated radioisotope and to concentrate samples for electrophoresis. The corresponding cell layers were then washed 3 times with cold PBS, lysed in PAGE-SDS sample buffer and boiled for 5 minutes to inactivate proteinases. The aliquots of concentrated medium, cell lysates (from equal cell number) and procollagen I purified from human skin fibroblast cells (gift from W. Arnold) were electrophoresed on 6% polyacrylamide-SDS gels with 3% stacking regions. The gels were fixed for 30 minutes in 10% acetic acid, 20% methanol, dried and exposed to film at -70°C for 7 days.

*c-myc* antisense treatment decreased in secreted [$^{14}$C] proline labeled procollagen and was similar to the Western blot DNA. *c-myc* antisense treatment

significantly increased the intracellular concentration of procollagen I compared to untreated cells and cells treated with a scrambled oligonucleotide (FIG. 19).

*B. The Increase In Intracellular Procollagen Level Is Not Due To The Inability Of Human Smooth Muscle Cells To Hydroxylate Proline After c-myc Antisense Oligonucleotide Treatment.*

The intracellular accumulation of procollagen may reflect the inhibition of post-translational modifications required for the assembly of procollagen $\alpha$ chains into a triple-helical conformation. Therefore, to rule out this possibility, the affects *c-myc* antisense treatment of prolyl 4-hydroxylase activity and hydroxyproline content were measured.

Post-confluent smooth muscle cells were incubated with or without oligonucleotides in 20% FBS-DMEM for 24 hours as described above. Cell layers were washed with cold PBS 3 times and lysed in 0.2 M NaCI, 20 mM Tris-HCl, pH 7.4, 0.1% Triton x-100, 0.1 M glycine and 10 $\mu$M DTT (the methods of K.J. Kivirikko, *et al.*, Methods in Enzymology, 82:245-364 (1982) are incorporated by reference herein). The lysates were then sonicated for 30 seconds on ice and protein concentration was measured. The remaining cell lysates were centrifuged at 13,000 rpm for 20 minutes at 4°C.

The aliquots of supernatant were assayed for prolyl 4-hydroxylase activity by measuring the formation of radioactive 4-hydroxyproline using a [$^{14}$C] proline-labeled procollagen I as substrate (the methods of K.J. Kivirikko, *et al.*, Anal. Biochem., 19:249-255 (1967) are incorporated by reference herein). The aliquots of cell lysate were hydrolyzed, hydroxyproline was assayed by a specific chemical procedure.

Prolyl 4-hydroxylase activity, a pivotal enzyme controlling triple helix formation, was similar among experimental subgroups. It was 11.7$\pm$0.1 (dpm/$\mu$g protein, mean $\pm$ SD of 2 separate experiments) in control smooth muscle cells treated with no oligonucleotides. 12.$\pm$2.3 in cells incubated with scrambled oligonucleotides (16 $\mu$M) and 10.5$\pm$2.0 after treatment with *c-myc* antisense oligonucleotides (16 $\mu$M).

Hydroxyproline content was greater in *c-myc* antisense (SEQ ID No:1) cells than in untreated or scrambled oligonucleotide treated cells (FIG. 20). The increase in hydroxyproline content reflects the increase in procollagen levels, which is a substrate for prolyl 4-hydroxylase.

**EXAMPLE 13.** INHIBITION OF EXTRACELLULAR MATRIX PRODUCTION IN HUMAN FIBROBLASTS HUMAN SKIN FIBROBLASTS CELLS USING *C-MYC* ANTISENSE OLIGONUCLEOTIDES

This example further demonstrates the effectiveness of *c-myc* antisense oligonucleotide treatment to inhibit the synthesis of extracellular martrix protein by human skin fibroblast cell, in particular procollagen I and III. Antisense treatment greatly reduced extracellular procollagen levels in stimulated human skin fibroblast cells (treated with 20% FBS). This example also further confirms the findings of Example 7.

*A. Assay of Extracellular and Intracellular collagen I and III Using Western Blots*

To further determine whether *c-myc* antisense oligonucleotides inhibited the synthesis of extracellular and intracellular procollagen I and III in human skin fibroblasts, procollagen levels were determined, as discussed for human smooth muscle cells in Example 9 and 12. Human skin fibroblasts cells used in the human skin fibroblast experiments were confluent. Cells were obtained and cultured as in Example 7 and by methods known in the art.

*B. Rapid Inhibition of Extracellular Collagen I and III Secretion By c-myc Antisense Oligonucleotide Treatment*

These experiments show that *c-myc* antisense oligonucleotides inhibit the secretion of extracellular collagen I and III by human skin fibroblasts in a dose-dependent, antisense sequence dependent manner and with a rapid time course of inhibition.

To further determine the dose response and antisense specificity of *c-myc* antisense oligonucleotides for inhibition of extracellular matrix production, extracellular collagen I was measured using Western blots of human fibroblast cell protein isolated from human fibroblasts subjected to different treatments. Human

fibroblast cells were exposed to either scrambled oligonucleotides (SEQ ID No:4; 16 $\mu$M), mismatched oligonucleotides (SEQ ID No:3; 16 $\mu$M), antisense oligonucleotides (SEQ ID No:1, 4, 8 and 16 $\mu$M) or no oligonucleotides for 24 hours as discussed herein and the proteins of those cells were assayed for collagen I in the Western blot shown in FIG. 21, lanes 1-5, respectively. The double band reflects collagen $\alpha_1$(I) and $\alpha_2$(I) chains, upper and lower bands, respectively. Antisense treated cells showed a proportional decrease of the amount of collagen I with increasing levels of *c-myc* antisense oligonucleotide, while the control treatments (no treatment scrambled and mismatch) showed higher amounts of collagen than the lowest concentration of *c-myc* antisense oligonucleotide used. Oligonucleotide (SEQ ID No:1; 16 $\mu$M) also inhibited cell proliferation in freshly plated fibroblasts; compared to 20% FBS and sense controls, cell proliferation was inhibited approximately 25 and 50 percent after four days, respectively. These results taken together with the other results discussed herein, especially the lack of up regulation of collagen and procollagen degradation pathways by *c-myc* antisense treatment, show that extracellular matrix production, in particular procollagen I secretion is inhibited by *c-myc* antisense treatments.

The time course of *c-myc* antisense oligonucleotide treatment of human skin fibroblast cell collagen I secretion showed rapid inhibition of secretion of de novo synthesized collagen I. At time zero human skin fibroblast cells were exposed to fresh medium (as used herein "fresh medium" is medium containing 20% FBS and ascorbic acid 50 $\mu$g/ml and other components as indicated) with either no oligonucleotides, scrambled oligonucleotides, or antisense oligonucleotides for 24 hours (concentration of the oligonucleotides was 16 $\mu$M; SEQ ID Nos:1 and 3). At three, six and twenty four hours aliquots of each cell medium were taken and assayed for the presence of collagen I by Western blot, as discussed herein. A reduction in collagen I level was observed in the antisense treated cells compared to the control cells (untreated and scrambled treated) in as early as three hours. At twenty four hours the antisense treated cells showed at least 5 to 10 times less secreted collagen I than the control cells. The six hour value produced intermediate results.

The secretion of collagen III by human fibroblast cells was also inhibited by *c-myc* antisense oligonucleotides. Human fibroblast cells were treated with fresh medium containing either no oligonucleotides, 16 μM scrambled oligonucleotides or 16 μM *c-myc* antisense oligonucleotides for twenty four hours and the medium was assayed for the presence of extracellular collagen III at the end of the twenty four hours. No extracellular collagen III was observed in the antisense treated cells, while the control cells (no treatment and scrambled treated) showed the presence of extracellular collagen III (at least 10-100 times higher level of collagen III, as compared to the antisense treat cells).

Thus, these results taken together with the other results discussed herein, show that extracellular matrix production, in particular procollagen I and III secretion, is inhibited by *c-myc* antisense treatments.

*C. Accumulation Of Intracellular Procollagen I And Simultaneous Decrease In Extracellular Procollagen I By c-myc Antisense Oligonucleotide Treatment*

Inhibition of procollagen secretion by *c-myc* antisense treatment of human fibroblast cells was associated with an accumulation of intracellular procollagen I. Human fibroblast cells were treated with fresh medium containing either no oligonucleotides, 16 μM scrambled oligonucleotides, or 16 μM *c-myc* antisense oligonucleotides for twenty four hours and the medium and the corresponding human fibroblast cells were assayed for the presence of extracellular procollagen I and intracellular procollagen I using $^{14}C$ proline labelled protein synthesis products separated as discussed herein. In FIG. 22, the major bands in lanes 1-3 show procollagen $\alpha_1$(I, III) and $\alpha_2$(I) chains. The amount of secreted procollagen I is reduced at least 10-20 times in the antisense treated cells compared to the control cells (no treatment, scrambled treated, lanes 1 and 2, respectively). The upper band in lanes 1-3 is approximately 116 Kd ("upper band"), which corresponds to procollagen $\alpha_1$(I, III); the lower band in lanes 1-3 (from left to right) is approximately 100 Kd, which corresponds to $\alpha_2$(I). In FIG. 22, lanes 4-6 show intracellular procollagen I is at least five times greater in antisense treated cells (lane 6) than the control cells (lanes 4 and 5). In FIG. 22, lane 7 shows the molecular weight markers.

Thus, these results demonstrate that *c-myc* antisense treatment decreases the amount of procollagen secreted by human fibroblast cells and increases the amount of procollagen in the cells.

**EXAMPLE 14.** *C-MYC* ANTISENSE OLIGONUCLEOTIDES DID NOT CHANGE THE THERMAL STABILITY OF INTRACELLULAR PROCOLLAGEN IN HUMAN SKIN FIBROBLASTS

The tertiary structure of intracellular procollagen was not changed by *c-myc* antisense treatment. Human fibroblast cells were treated with fresh medium containing either no oligonucleotides, 16 $\mu$M scrambled oligonucleotides, or 16 $\mu$M *c-myc* antisense oligonucleotides for twenty four hours. The melting temperature of procollagen from each experimental condition was measured as discussed herein and known in the art. FIG. 23 shows that the melting profile for control cells (untreated and scrambled treated) and antisense treated are similar. These results demonstrate that the procollagen present inside of antisense treated cells maintains a normal triple-helical conformation that is the same as in control treated cells.

Thus, *c-myc* antisense treatment does not lead to a detectable alteration of procollagen structure that could lead to increased intracellular degradation or decreased protein export from the cell.

**EXAMPLE 15.** *C-MYC* ANTISENSE OLIGONUCLEOTIDE TREATMENT DID NOT CHANGE THE PROCOLLAGEN $A_1$(I) MRNA LEVEL IN HUMAN SKIN FIBROBLASTS

This example demonstrates that *c-myc* antisense oligonucleotide treatment does not inhibit the synthesis of extracellular martrix protein by human skin fibroblast cells, in particular procollagen I, by reducing the mRNA level of procollagen. Antisense treatment did not reduce mRNA procollagen levels in stimulated human skin fibroblast cells (treated with 20% FBS).

*A. Measurement of Procollagen $\alpha_1$(I) mRNA*

RNA was isolated and Northern analysis was carried out as follows: Cells were washed three times with cold PBS buffer and lysed in 4 M guanidinium isothiocyanate. RNA was extracted by phenol/chloroform and quantitated by

spectrophotometric absorbance at 260/280 nm. RNA samples (20 $\mu$g) were separated by electrophoresis on 0.8% agarose-formaldehyde gels. Following transfer of the RNA to nitrocellulose filters, blots were fixed by ultraviolet irradiation. The blots were prehybridized in 5x standard saline citrate, 2x Denhardt's solution, O.1% SDS and 0.2mg/ml of denatured salmon sperm DNA for at least 4 hours at 65°C. Hybridization was performed overnight using the same buffer containing cDNAs radio labeled with $^{32}$P dCTP by nick translation to a specific activity of approximately 108-109 cpm/$\mu$g. Blots were washed at 65°C in 0.5x standard saline citrate and O.1% SDS. The filters were exposed to Kodak X-Omat film at -70°C with intensifying screens for 1-5 days. The autoradiography of the resulting Northern blots was quantitated by scanning densitometry and integration of peak areas. The probes used for this study were as follows: a 1.8 Kb pro-alpha 1(1) (HF-677) cDNA corresponding to the COOH-terminal propeptide and the carboxy-terminal portion of the triple helical region of human pro-alpha 1(1) chain of type I procollagen (Chu *et al.*, *Nucleic Acids Res.* 10:5925-5934, 1982).

*B. Procollagen $\alpha_1$(I) mRNA Levels Are Not Decreased By c-myc Antisense Treatment*

The mRNA level of procollagen alpha I in human fibroblast cells was unaffected by *c-myc* antisense treatment. Human fibroblast cells were treated with fresh medium containing either no oligonucleotides, 16 $\mu$M scrambled oligonucleotides, or 16 $\mu$M *c-myc* antisense oligonucleotides for twenty four hours. mRNA was purified from each experimental condition and analyzed for the presence of procollagen alpha I mRNA, as discussed herein and known in the art. FIG. 24 shows that control cell procollagen I mRNA (untreated and scrambled treated; lanes 1 and 2, respectively) levels are similar to the antisense treated cell procollagen alpha I mRNA levels (lane 3). These results demonstrate that *c-myc* antisense treatment of human fibroblast cells does not down regulate mRNA transcription of the procollagen alpha I gene.

Thus, taken together with the other results discussed herein, the mRNA results show that *c-myc* antisense treatment decreases the secretion of procollagen I

and III, which are proteins that lead to the synthesis of collagen in the extracellular matrix.

**EXAMPLE 16.** *C-MYC* ANTISENSE OLIGONUCLEOTIDES DID NOT ALTER THE RECOVERY OF THE SYNTHESIS OF EXTRACELLULAR MATRIX PROTEIN AFTER THE CESSATION OF *C-MYC* ANTISENSE OLIGONUCLEOTIDE TREATMENT OF HUMAN SKIN FIBROBLASTS

The ability of human fibroblast cells to synthesize collagen I after removal of *c-myc* antisense oligonucleotide treatment was not impaired. Human fibroblast cells were treated with fresh medium containing either no oligonucleotides, 16 $\mu$M scrambled oligonucleotides, or 16 $\mu$M *c-myc* antisense oligonucleotides for twenty four hours. Human fibroblast cells from each experimental condition were then exposed to fresh medium without any oligonucleotide additions and assayed for the presence of extracellular collagen I, as discussed herein. FIG. 25 shows that control cells (untreated and scrambled pretreated) and antisense treated cells produced similar levels of extracellular collagen I twenty four hours after abatement of the treatment. These results demonstrate that after twenty four hours there was full recovery of collagen production in *c-myc* antisense treated human fibroblast cells.

Thus, *c-myc* antisense treatment did not produce any cytotoxic effects that resulted in the inability of the cell to return to pretreatment secretion levels of procollagen.

**EXAMPLE 17.** INHIBITION OF SYNTHESIS OF EXTRACELLULAR MATRIX IN PIG CORONARY ARTERIES

This example further demonstrates the *in vivo* effectiveness of localized *c-myc* antisense oligonucleotide treatment to decrease the progression of extracellular matrix synthesis after tissue trauma. This example also further demonstrates the *in vivo* effectiveness of *c-myc* antisense treatment in decreasing the progression of internal organ scaring, as well as decreasing the progression of tissue scaring associated with the synthesis of extracellular matrix molecules, such as collagen.

*A. Inhibition of The Production of Extracellular Matrix Fibrillar Material By c-myc Antisense Oligonucleotide Treatment*

To determine the effects of *c-myc* antisense treatment on the progression of extracellular matrix synthesis after tissue trauma, pig hearts were injured *in vivo*, treated with oligonucleotides as discussed in Example 8 (unless otherwise indicated), and sacrificed at three days, seven days, and twenty eight days after the delivery of oligonucleotides. The same *c-myc* antisense and sense oligonucleotides (SEQ ID No:1 and 2) and dosages (1 mg per artery) were used as in Example 8.

Cross-sections of traumatized (injured) coronaries from antisense treated pigs showed differences in microscopic extracellular architecture compared to the sense treated traumatized coronaries. Traumatized coronaries from each experimental group were examined as blind samples with light microscopy, magnification 20-62X. Tissue was stained with Verhoff-Van-Gieson stain, as known in the art, to highlight cells and extracellular matrix, and sirus red stain, as known in the art, to highlight collagen. The *c-myc* antisense treated vessels, which displayed a smaller neointima area compared to control vessels, also displayed less red staining than the control vessels (sense treated). The antisense treated coronaries showed less extracellular fibrillar material compared to the sense treated coronaries; and the antisense treated coronaries showed less extracellular space between cells. Neointimal smooth muscle cell proliferation was also assessed using immunostaining for PCNA (Proliferating Cell Nuclear Antigen) following balloon denudation and transcatheter delivery of *c-myc* sense (S) or antisense (AS) oligonucleotides (1 mg) into porcine coronary arteries, see Table IV. Antisense treatment of coronaries inhibited the progression of smooth muscle cell proliferation caused by tissue trauma compared to sense treatment of coronaries. PCNA staining permitted the assessment of the number of proliferating cells in a given neointima cross-section. Generally 200-1,000 cells were counted and the percentage of proliferating smooth muscle cells determined and expressed as the proliferative index. The proliferative index (PI), neointima/media (I/M) ratio and the reduction in I/M ratio ($\Delta$I/M) were as follows:

TABLE IV

| Day | $R_x$ | n | P*(%) | I/M ratio | ΔI/M(%) |
|---|---|---|---|---|---|
| 3 | S | 4 | 5±2 | 0.09±0.03 | |
| | AS | 8 | 3±5 | 0.05±0.02 | ↓44 |
| 7 | S | 4 | 47±13 | 0.28±0.04 | |
| | AS | 8 | 11±13** | 0.12±0.05** | ↓57 |
| 28 | S | 6 | N/A | 1.19±0.51 | |
| | AS | 4 | N/A | 0.47±0.30* | ↓61 |

P* - proliferating cells/total number of cells x 100, *p(0.05, **p(0.01

Coronaries with a reduction of the I/M ratio have an improved lumenal diameter. These results demonstrate that localized *c-myc* antisense treatment inhibits smooth muscle cell growth and extracellular matrix formation over time.

Thus, localized *c-myc* antisense treatment inhibits the progression of scar formation in a traumatized tissue, such as scar formation in coronaries after balloon angioplasty trauma caused by smooth muscle cell proliferation and extracellular matrix formation.

**EXAMPLE 18.** RAPID LOCALIZATION *C-MYC* ANTISENSE OLIGONUCLEOTIDES IN PIG CORONARY ARTERIES

This example demonstrates the *in vivo* effectiveness of *c-myc* antisense oligonucleotides in penetrating vascular and connective tissues using local delivery devices. In conjunction with the other results discussed herein, these results demonstrate the ability of *c-myc* antisense treatment to localize in cell nuclei within 30 minutes and before the onset of *c-myc* induction (which occurs over three to four hours). The local distribution of *c-myc* antisense oligonucleotides after local administration to the vessel wall of coronary arteries decreases scar formation following balloon angioplasty.

*A. Local Delivery Of c-myc Antisense Oligonucleotides Labelled With Either Carboxyfluorscein Or $^{35}S$ Phosphothioate*

The *c-myc* antisense oligonucleotides used in this Example were prepared as discussed herein. Phosphorothioate antisense oligonucleotides (SEQ ID No:1)

directed against the translation initiation region of the human c-*myc* gene used in the distribution studies labeled with carboxyfluorosceins (480 nm excitation, 520 nm emission, Molecular Probes, Inc.) as previously described in Iverson, *et al*, *Antisense Res.* Dev. 2: 211-222 (1992), the methods of which are herein incorporated by reference. For the quantitative studies oligonucleotides were $^{35}S$-labeled at each internucleotide link with the specific activity of $1.8 \times 10^8$ cpm/$\mu$mol.

Domestic crossbred pigs (*Sus scrofa*) were used and prepared for experiments as discussed herein. A coronary ostia was cannulated using a 9 French SAL 1 guiding catheter (Cordis Corp., Miami, FL) was under fluoroscopic guidance. After intracoronary injection of nitroglycerin (100 $\mu$g), a baseline coronary angiography was obtained. Selected portions of the coronary arteries were then subjected to denudation (traumatization) with an oversized balloon inflated 3 times (6 atm) for 30 sec. Then, the delivery device, a Kaplan-Simpson Infusasleeve (of LocalMed, Palo Alto, CA) was advanced over a standard angioplasty balloon ("support" balloon) to the region of interest. This device consists of multilumen infusion region (18 mm) containing multiple sideholes (40 $\mu$m) which are juxtapositioned against the vessel wall by inflating a support balloon. The device used in studies infuses solutions using rigid, noncollapsable channels. This design minimizes drug loss to the bloodstream and permits separate control of drug infusion and placement of the drug delivery device in the vessel. After a full expansion of the internal balloon catheter, the infusion portion was fluoroscopically ascertained, c-*myc* antisense oligonucleotides were administered. Intramural injections were carried out under 100 PSI of the external pressure. The total volume of the injectate was 5-6 ml, delivered by the external pump at 40 ml/min. The administered dose was 1 mg per artery. After injection was completed, devices were removed and final angiograms were recorded. To verify proper conditions of oligonucleotides delivery, serial quantitative coronary angiography was employed to measure the initial luminal diameter of selected coronary arteries, luminal diameter prior to oligonucleotides delivery (i.e., after denuding injury) and after oligonucleotides administration. In addition, diameters of denudation and support balloons were measured *in situ*. Guiding catheter was

used as a scaling device for all angiographic measurements. At the indicated times, the animals were given a lethal dose of sodium pentobarbital (100 mg/kg iv).

As shown in Table V, mean luminal vessel diameter was 2.5 ± 0.1 mm at baseline. The coronary arterial injury was produced with the oversized balloon (also known as denuding) (mean balloon:artery ratio 1.1). All vessels remained patent following balloon overstretch with mean luminal diameter of 2.6 ± 0.1 mm. C-*myc* antisense oligonucleotides were administered through expanded delivery device achieving direct contact with the endoluminal surface or the vessel (mean balloon:artery ratio 1.0).

**Table V**

**Angiographic Characteristics Based On Quantitative Coronary Angiography (n=14).**

| | |
|---|---|
| **Initial luminal diameter (mm)** | **2.5 ± 0.1** |
| Denuding balloon/artery ratio | 1.1 ± 0.0 |
| **Post-denudation luminal diameter (mm)** | **2.6 ± 0.1** |
| Delivery balloon/artery ratio | 1.0 ± 0.0 |
| **Post-delivery luminal diameter (mm)** | **2.6 ± 0.1** |

*B. Qualitative Assessment of oligonucleotides Delivery*

To determine distribution of fluorescent-labeled oligonucleotides after transcatheter delivery, the coronary arteries were excised with adjacent tissues. After the vessel lumen was flushed with PBS, the arteries were fixed in 10% buffered formalin for 6 hours and embedded in paraffin. The arteries were cut into serial 4-μm-thick sections (50 mm per vessel including 18 mm injected portion. The examination of the site of injection as well as proximal and distal portions of the vessel provided information regarding the longitudinal oligonucleotides distribution. In addition, noninjected coronary artery from the same animal served as the additional control. The sections were deparaffinized end covered with SlowFade-Light antifade reagent (Molecular Probes, Inc.,

Eugene, OR). Each slide contained the vessel, periadventitial tissue and adjacent myocardium (FIG. 17) which allowed to determine the depth of oligonucleotide penetration. The fluorescent-labeled antisense oligonucleotides were visualized under Nikon Optiphot microscope equipped for epifluorescence with XF-22 filter. Photographs were taken on Kodak Ektachrome P1600 films.

To analyze intracellular localization of fluorescent labeled oligonucleotides *in vitro* and *in vivo,* laser scanning confocal microscope (model Zeiss Axiovert 100) adapted with MRC-600 krypton-argon laser (Bio-Rad) was used. The krypton-argon laser emits laser lines at excitation wavelength 488 nm. The induced fluorescent light is scanned through a 63x objective and converted to a video signal for display on a computer screen. The images were photographed off the computer screen on Kodak Ektachrome 100 films. For *in vitro* studies, in order to determine the time course of oligonucleotides uptake by vascular smooth muscle cells, cells were grown on chamber slides in medium containing 10% fetal bovine serum for 2 days and fluorescein-labeled oligonucleotides were added (8 $\mu$M) for 2, 30 minutes and 2 hours. Then, cells were washed 3 times with PBS and fixed in 50% acetone and 50% methanol. For *in vivo* studies, sections of the coronary arteries obtained as described above were analyzed by confocal microscopy at 0.4 - 0.5 $\mu$m optical thickness.

C. *Distribution Of Antisense Oligonucleotides In The Vessel Wall*

To determine oligonucleotide distribution in the arterial wall, their localization in the coronary arteries was examined at 30 minutes and 3 days after intramural administration (n=4). Fluorescein-labeled oligonucleotides were detected only in the region of injection but not in distal segments or in non-injected coronary arteries. At 30 minutes, three distinct focal patterns of oligonucleotides distribution were noted within injected segments which had previously undergone balloon denudation. Dense *transmural* localization involving the entire thickness of media was associated with more sparse adventitial and perivascular distribution (FIGS. 27A and 27B). In each section, oligonucleotides occupied only the portion of the arterial circumference. *Subintimal nontransmural* pattern was common; it was either interspersed between transmural localization or at the edges of injected segments (FIGS. 27C and 27D). *Midwall nontransmural*

pattern was the least common and it was found adjacent to transmural localization (FIGS. 27E and 27F). The vessel adventitia was devoid of antisense oligonucleotides in sections exhibiting nontransmural distribution.

The role of preceding vascular damage in *c-myc* antisense oligonucleotide distribution in the arterial wall was also examined. The analysis of consecutive slides revealed that transmural distribution was the most common in the areas of dissection with oligonucleotide localization extending to immediately adjacent sections. The subintimal nontransmural pattern was usually associated with preserved vascular integrity. At 3 days, oligonucleotides were clearly visible in transmural localization (FIGs. 28A and 28B). The subintimal nontransmural distribution was less evident likely as a result of oligonucleotides washout. No evidence for linear "jet-like" localization was present either at 30 minutes or 3 days.

These data collectively point to a focal pattern of intramural distribution of oligonucleotides after pressure-driven transcatheter administration. Coronary artery dissection due to prior balloon denudation appeared to facilitate the entry and transmural distribution of oligonucleotides. The nontransmural pattern was often associated with the intact internal elastic lamina.

*D. Intramural Trafficking Of Oligonucleotides In Coronary Arteries*

The residence time of oligonucleotides *in situ* was measured to determine if antisense treatment led to oligonucleotide retention. Accordingly, to quantitatively address this issue, $^{35}$S-labeled c-*myc* antisense oligonucleotides were delivered using a transcatheter in porcine coronary arteries. As shown in FIG. 29, at 30 min following site-specific administration. tissue radioactivity was measured which reflects an oligonucleotide content of 2510 $\pm$ 1436 cpm (n=6) in the vessel wall and 2163 $\pm$ 966 cpm (n=6) in the perivascular tissue as opposed to tissue background of 17 $\pm$ 4 cpm. Three days later, oligonucleotide-associated radioactivity increased to 3595 $\pm$ 1672 cpm (n=4) in the vessel wall, whereas in the periadventitial tissue levels decreased to 432 $\pm$ 201 cpm (n=4). The above sustained level of oligonucleotides was achieved despite a rapid plasma clearance ($t_{1/2}$ 15 $\pm$ 2 min). The amount of detected oligonucleotides both at 30 min and 3 days corresponded to about 0.1% of total administered dose. These results

indicate that pressure-driven transcatheter delivery leads to antisense oligonucleotides deposition in medial, and adventitial tissues, as well as, perivascular tissues.

*E. Vascular Integrity After Intramural Administration of oligonucleotides*

Since pressure-driven transcatheter injection of any therapeutic agent to the arterial wall may induce vascular damage the extent of damage was measured. *First,* using qualitative coronary angiography, non-flow limiting coronary dissection was observed in 1 out of 14 intramural injections in porcine coronary arteries notwithstanding prior injury with the oversized balloon. *Second,* we have utilized quantitative coronary angiography to assess luminal diameter following intramural oligonucleotides delivery (n=14). There was no significant change in luminal diameter after transcatheter oligonucleotides administration (Table V). *Third,* we have assessed the effects of transcatheter oligonucleotides injection using light microscopy. To this end, sections of coronary arteries with transmural distribution of oligonucleotides and no visible dissection were evaluated in order to determine subtle changes in cellular and extracellular matrix components of the vessel wall. As shown in FIG. 30, transmural distribution of oligonucleotides (FIG. 30A) was associated with the intact internal elastic lamina and elastic tissues throughout the vessel wall (FIG. 30B). A mild decrease in cytoplasmic staining and nuclear pyknosis was occasionally noted at the site of oligonucleotides retention at 30 min (FIG. 30C). These changes were not found at 3 days after the delivery (FIG. 30D).

**EXAMPLE 19.** RAPID NUCLEAR LOCALIZATION OF *C-MYC* ANTISENSE OLIGONUCLEOTIDES IN *IN VITRO* HUMAN SMOOTH MUSCLE CELLS AND *IN VIVO* PORCINE SMOOTH MUSCLE CELLS

This example further demonstrates the effectiveness of *c-myc* antisense oligonucleotide treatment *in vivo* by showing the rapid localization of *c-myc* antisense oligonucleotides in the nuclei of smooth muscle cells both *in vitro* (human) and *in vivo* (porcine). This rapid time course allows for inhibition of *c-myc* gene activation, previously established on the order of hours (3 to 4 hours).

A. *Intracellular Localization Of Carboxyfluoroscein labelled c-myc Antisense Oligonucleotides*

To analyze intracellular localization of fluorescent labeled oligonucleotides *in vitro*, laser scanning confocal microscope (model Zeiss Axiovert 100) adapted with MRC-600 krypton-argon laser (Bio-Rad) was used. The krypton-argon laser emits laser lines at excitation wavelength 488 nm. The induced fluorescent light is scanned through a 63x objective and converted to a video signal for display on a computer screen. The images were photographed off the computer screen on Kodak Ektachrome 100 films. For *in vitro* studies, in order to determine the time course of oligonucleotides uptake by vascular smooth muscle cells, cells were grown on chamber slides in medium containing 10% fetal bovine serum for 2 days and fluorescein-labeled oligonucleotides were added (8 $\mu$M) for 2, 30 minutes and 2 hours. Then, cells were washed 3 times with PBS and fixed in 50% acetone and 50% methanol.

B. *Cellular Uptake of Antisense oligonucleotides*

The intramural delivery of antisense oligonucleotides should be followed by a rapid cellular uptake of oligonucleotides in order to permit the inhibition of expression of targeted gene, in this case *c-myc*. As illustrated on FIG. 31, vascular smooth muscle cells demonstrate *in vitro* preferential nuclear localization of fluorescence 30 min following the onset of incubation with fluorescein-labeled oligonucleotides. Likewise, at 30 min following *in vivo* transcatheter administration in pigs, nuclear localization of oligonucleotides was noted within the media (FIG. 32) and the adventitia (FIG. 33) which demonstrates comparably rapid uptake of antisense oligonucleotides *in vivo*. Accordingly, intracellular uptake of antisense oligonucleotides by vascular smooth muscle cells *in vitro* and *in vivo* occurs during a critical time window available for the modulation of c-*myc* proto-oncogene, an important inducible gene regulating smooth muscle cells proliferation following vessel wall injury.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Yi Shi and Andrew Zalewski

(ii) TITLE OF INVENTION: Arteriovenous and Venous Graft Treatments: Methods and Compositions

(iii) NUMBER OF SEQUENCES: 11

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch diskette
(B) COMPUTER: IBM compatible
(C) OPERATING SYSTEM: Windows 3.1/DOS 5.0
(D) SOFTWARE: Microsoft Word for Windows, vers. 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: PCT Application US94/11853
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACGTTGAGG GGCAT 15

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGCCCCTCA ACGTT 15

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AACGTGGATT GGCAG 15

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAACGGAGAC GGTTT 15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TATGCTGTGC CGGGGTCTTC GGGC 24

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGGAGAGTCG CGTCCTTGCT 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCATGGAGCA CCAGGGGCTC 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCATGGAGCA CCAGGGGCTC 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGCCTTTTCA TTGTTTTCCA 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CATTGTTTTC CAACTCC 17

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TTCATTGTTT TCCAATT 17

WHAT IS CLAIMED IS:

1. A method for preventing failure of a hemodialysis access site of a hemodialysis patient, comprising:

contacting said hemodialysis access site with an oligonucleotide complementary to nuclear proto-oncogene mRNA.

2. A method of claim 1 wherein said hemodialysis access site is selected from the group consisting of Brescia-Cimino fistula, snuffbox fistula, brachiocephalic fistula, PTFE hemodialysis graft, and bovine carotid artery graft and autogenous grafts.

3. A method of claim 1 wherein said contacting comprises injection, catheterization or infusion.

4. A method of claim 1 wherein said contacting comprises diffusion from an implant, or diffusion from a reservoir located in perivascular tissue.

5. A method of claim 1 wherein said contacting comprises injection into the perivascular tissue either by perivascular injection prior to surgical site closure or percutaneous injection after closure of the surgical site.

6. A method of claim 5 wherein said contacting is repeated following construction of said hemodialysis access site.

7. A method for treating vascular grafts, comprising:
contacting said vascular graft with an oligonucleotide complementary to nuclear proto-oncogene mRNA.

8. A method of claim 7 wherein said contacting comprises diffusion from an implant, or diffusion from a reservoir located in perivascular tissue.

9. A method of claim 7 wherein said contacting comprises injection into the perivascular tissue either by perivascular injection prior to surgical site closure or percutaneous injection after closure of the surgical site.

10. A *ex vivo* method for preventing failure of vascular grafts made with veins, comprising:
contacting *ex vivo* said vein with an oligonucleotide complementary to nuclear proto-oncogene mRNA.

11. A method of claim 10 wherein said contacting comprises incubating said vein with said oligonucleotide for at least 10 minutes in a sterile and physiologically acceptable buffer.

12. A method of claim 11 wherein said physiologically accetable buffer is at 20 to 37 °C.

13. A method of claim 12 wherein said oligonucleotide has a concentration of 10 to 200 $\mu$M.

14. A method of claim 13 wherein said vein is selected from a group consisting of saphenous vein, cephalic vein, basilic vein, brachial vein, and femoral vein.

15. A composition, comprising:
an hemodialysis access site, and
an oligonucleotide complementary to nuclear proto-oncogene mRNA located in at least one layer of said hemodialysis access site.

16. A composition of claim 15 wherein said hemodialysis access site is selected from the group consisting of Brescia-Cimino fistula, snuffbox fistula, brachiocephalic fistula, PTFE hemodialysis graft, and bovine carotid artery graft and autogenous grafts.

17. A composition of claim 15 wherein said layer is selected from the group consisting of endothelial layer, media, adventitia and perivascular tissue.

18. A composition of claim 17 wherein said portion is selected from the group consisting a PTFE graft, a refillable reservoir, a stent, gel and a implantable device.

19. A composition of claim 15 wherein said oligonucleotide is located in a reservoir.

20. A composition of claim 19 wherein said reservoir contains between .5 and 10 mg of said oligonucleotide.

21. A composition of claim 20 wherein said reservoir is on the venous side of said hemodialysis access site.

22. A composition, comprising:
a vascular graft, and
an oligonucleotide complementary to nuclear proto-oncogene mRNA located in at least one layer of said vascular graft.

23. A composition of claim 22 wherein said graft is a prosthetic fistula, arteriovenous shunt, native vein fistula, venous grafts, immunosuppressed allografts, immunosupressed xenografts, and venous autografts.

24. A composition of claim 22 wherein said layer is selected from the group consisting of endothelial layer, media, adventitia and perivascular tissue.

25. A composition of claim 24 wherein said oligonucleotide has of a concentration of at least 10 $\mu$M in at least one said layer.

26. A composition of claim 25 wherein said oligonucleotide has of a concentration of at least 1 $\mu$M inside a cell of said layer.

27. A composition of claim 26 wherein said cell is either a smooth muscle cell, myofibroblast or a fibroblast.

28. A composition, comprising:
an excised human-compatible vein, and
an oligonucleotide complementary to nuclear proto-oncogene mRNA located in a vessel wall of said human-compatible vein.

29. A composition of claim 28 wherein said excised human-compatible vein is selected from the group of saphenous vein, cephalic vein, basilic vein, brachial vein, and femoral vein.

30. A composition of claim 28 wherein said oligonucleotide is located in a layer of said vessel wall selected from the group consisting of endothelial layer, media, and adventitia.

31. A composition of claim 30 wherein said oligonucleotide has of a concentration of at least 10 $\mu$M in at least one said layer.

32. A method of inhibiting the synthesis of extracellular matrix proteins in a human tissue comprising administering to the tissue a therapeutically effective amount of one or more antisense oligonucleotides each specific for a nuclear proto-oncogene.

33. The method of claim 32 wherein said nuclear proto-oncogene is selected from the group consisting of *c-myc, c-myb, c-Fos, N-myc, L-myc, p53, c-rel, c-ski, c-ets-1, and c-ets-2.*

34. The method of claim 33 wherein said nuclear proto-oncogene is selected from the group consisting of c-*myc* and c-*myb* and said extracellular matrix proteins are selected from the group consisting of collagen.

35. The method of claim 34 wherein said tissue is an arterial wall.

36. The method of claim 35 wherein said step of administering includes administering said antisense oligonucleotide locally with a catheter or a coated stent.

37. The method of claim 36 wherein said one or more antisense oligonucleotides consists of an antisense oligonucleotide specific for c-*myc* and an antisense oligonucleotide specific for c-*myb*.

38. The method of claim 37 wherein said one or more antisense oligonucleotides specific for said nuclear proto-oncogene are phosphorothioate oligonucleotides.

39. The method of claim 37 wherein said tissue comprises skin fibroblasts.

40. The method of claim 34 wherein said antisense compound is administered topically.

41. The method of claim 40 wherein said tissue comprises an internal organ containing smooth muscle cells or fibroblasts.

42. The method of claim 32 wherein said synthesis of extracellular matrix proteins is secretion of extracellular matrix proteins.

43. The method of claim 32 wherein said human tissue is connective tissue.

44. The method of claim 35 wherein said nuclear oncogene is *c-myc*.

45. The method of claim 44 wherein said extracellular matrix proteins is either procollagen I or procollagen III.

46. The method of claim 45 wherein said tissue comprises human fibroblasts.

47. The method of claim 45 wherein said tissue comprises human smooth muscle cells.

48. The method of claim 38 wherein said administering comprises local delivery using a catheter.

49. The method of claim 48 wherein said inhibiting of synthesis occurs in the absence of a biological process selected from the group consisting of down regulation of transcription of genes encoding extracellular matrix procollagen I and procollagen III, down regulation of mRNA translation of extracellular matrix procollagen I and procollagen III, up regulation of degradation intracellular pathways for extracellular matrix procollagen I and procollagen III precursors, and up regulation of degradation of extracellular matrix procollagen I and procollagen III.

50. A method of treating sclerotic disorders comprising the step of administering an effective amount of an antisense oligonucleotide specific for c-*myc*.

51. A method of inhibiting collagen synthesis in restenosis, the method comprising the steps of locally administering an effective amount of an antisense oligonucleotide specific for c-*myc*.

52. A method of claim 51 wherein said reducing of scar formation comprises decreasing the progression of extracellular matrix protein formation.

53. The method of claim 52 wherein said step of locally administering includes administering with a catheter or a coated stent.

54. A method of reducing scar formation in a human tissue comprising: administering to said human tissue a therapeutically effective amount of a proto-oncogene antisense oligonucleotide.

55. The method of claim 54 wherein said step of locally administering includes locally administering in combination an antisense oligonucleotide specific for c-*myc* and an antisense oligonucleotide specific for c-*myb*.

56. A method of inhibiting formation of fibrous connective tissue in a human, comprising:

administering to said human a therapeutically effective amount of a proto-oncogene antisense oligonucleotide.

57. A pharmaceutical composition useful for inhibiting the synthesis of extracellular matrix proteins, which composition comprises a pharmaceutically-acceptable excipient and an antisense oligonucleotide specific for a nuclear proto-oncogene that is present in an amount sufficient to inhibit the synthesis of extracellular matrix proteins when administered to a subject in need thereof.

58. The composition of claim 57 wherein said antisense oligonucleotide is chosen from the group of nucleotides consisting of Sequence ID numbers 1, 5, 6 and 7.

59. The method of claim 58 wherein said antisense oligonucleotide is chosen from the group of nucleotides consisting of Sequence ID numbers 1, 5, 6 and 7.

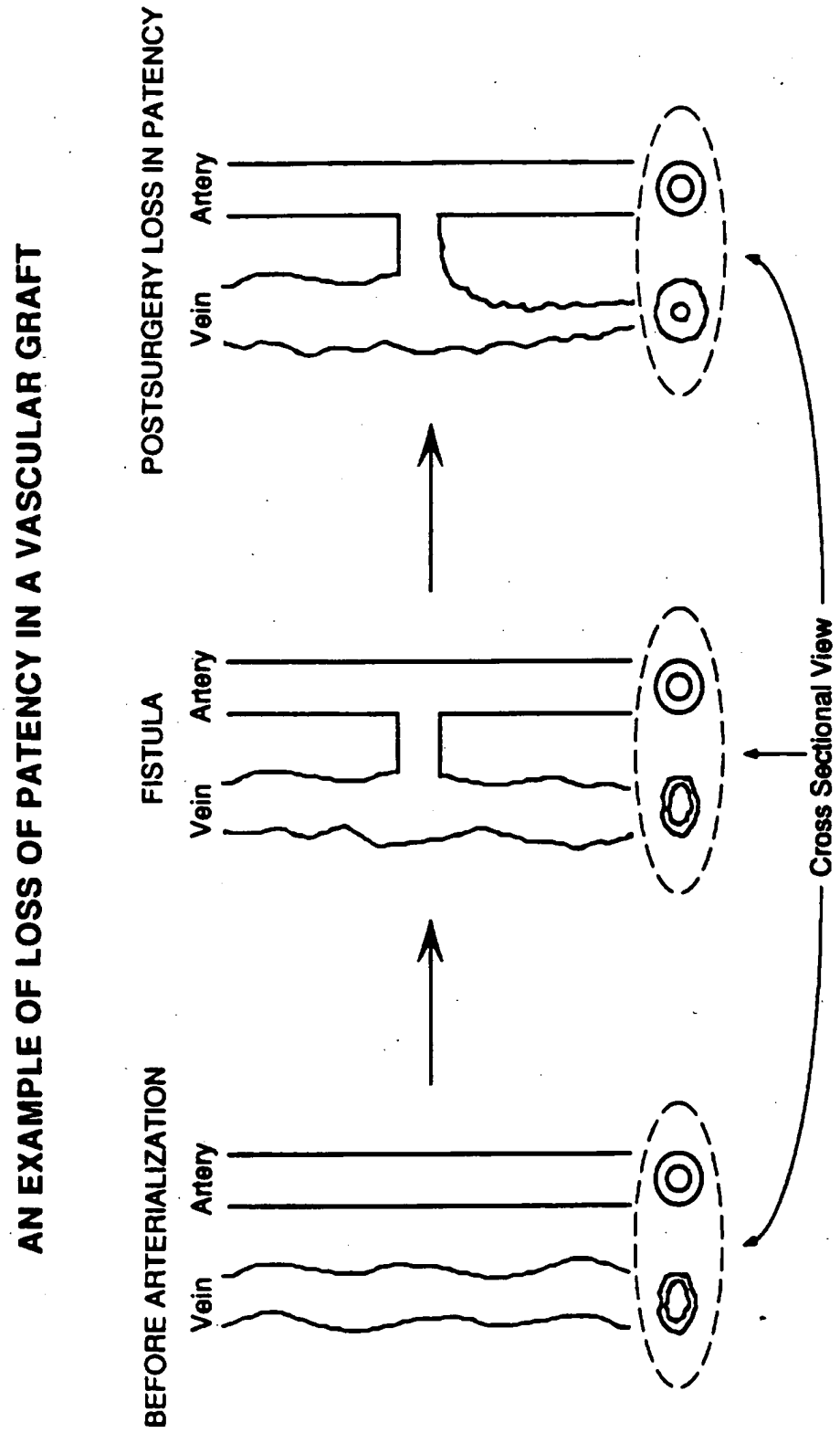
AN EXAMPLE OF LOSS OF PATENCY IN A VASCULAR GRAFT
POSTSURGERY LOSS IN PATENCY
Artery
Vein
FISTULA
Artery
Vein
BEFORE ARTERIALIZATION
Artery
Vein
Cross Sectional View

FIG. 1

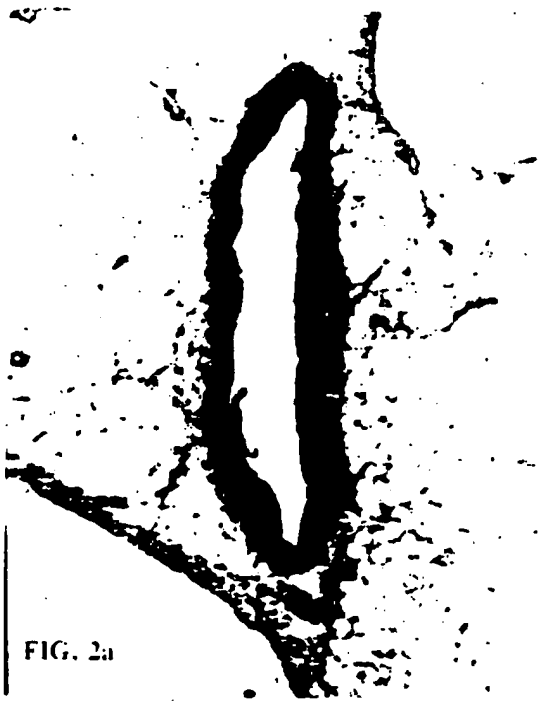

FIG. 2a

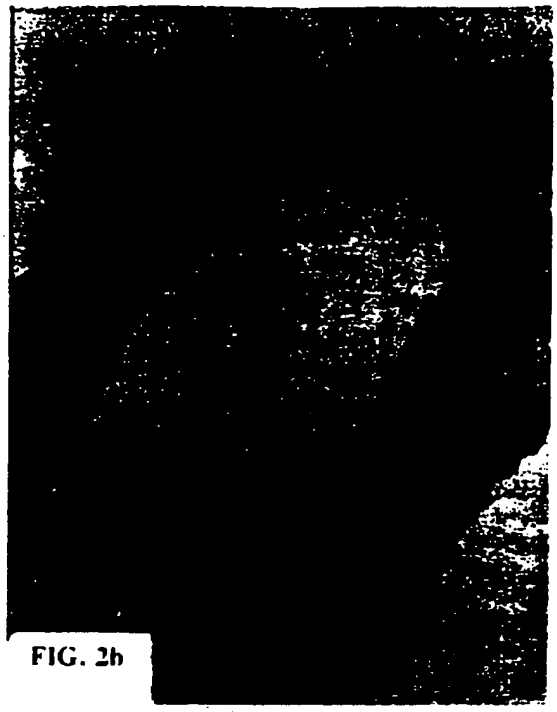

FIG. 2b

FIG. 3a

FIG. 3b

FIG. 4a

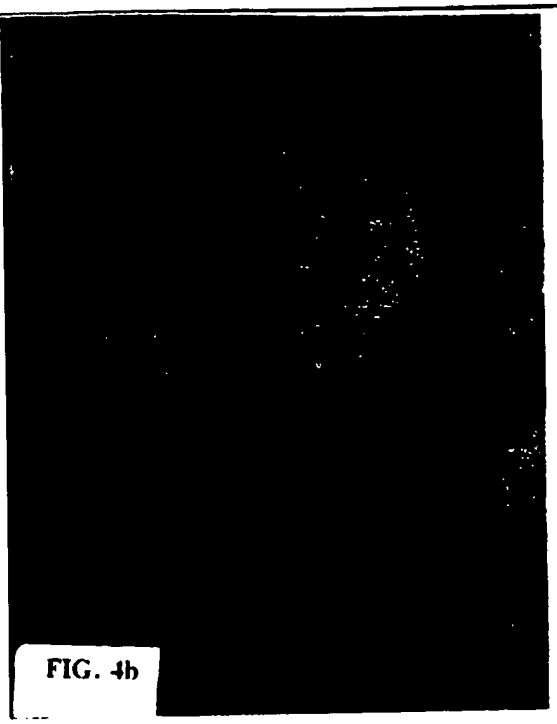

FIG. 4b

FIG. 4c

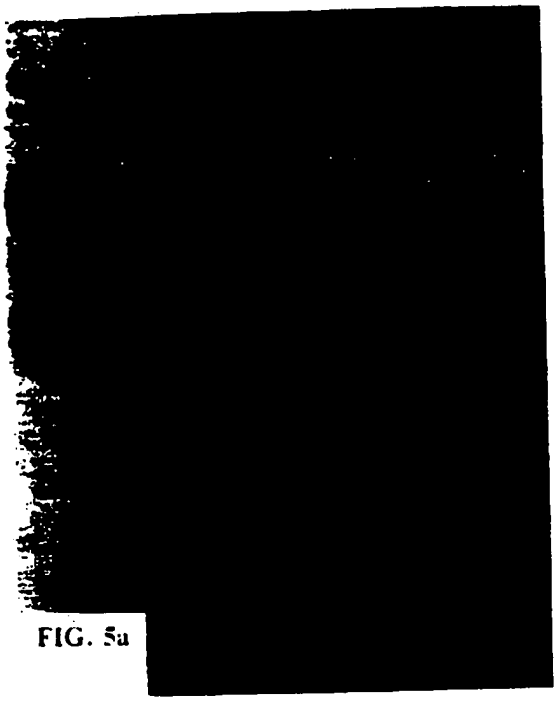

FIG. 5a

FIG. 5b

FIG. 5c

FIG. 6a

FIG. 6b

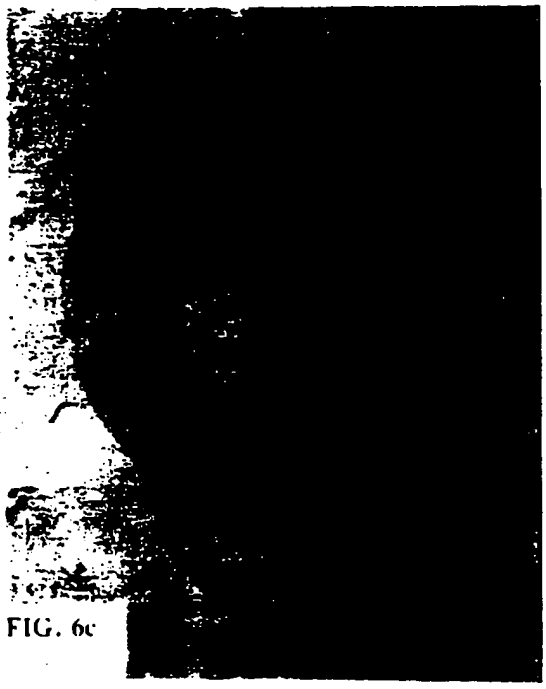

FIG. 6c

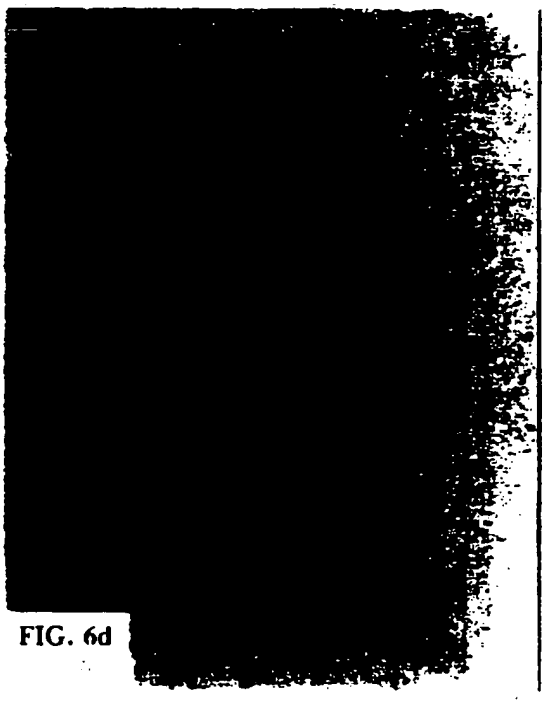

FIG. 6d

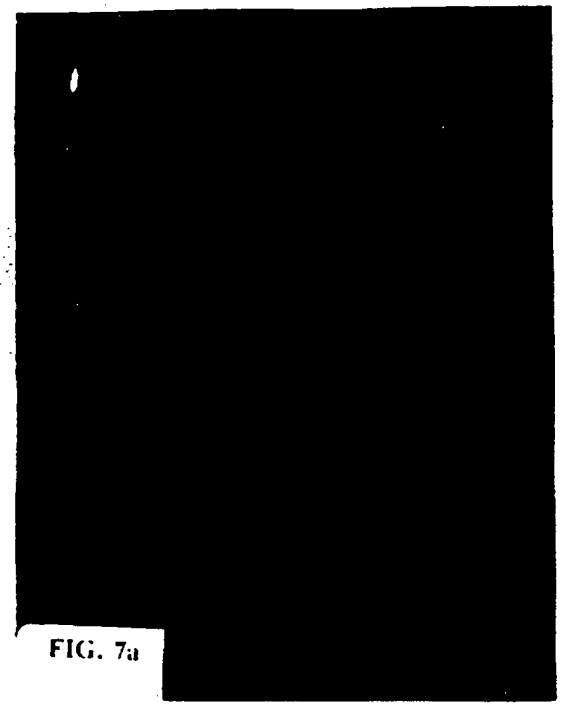

FIG. 7a

FIG. 7b

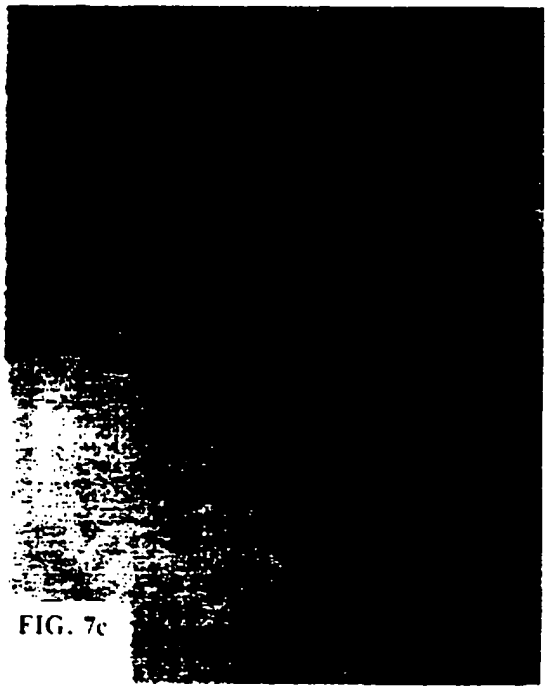

FIG. 7c

FIG. 8a

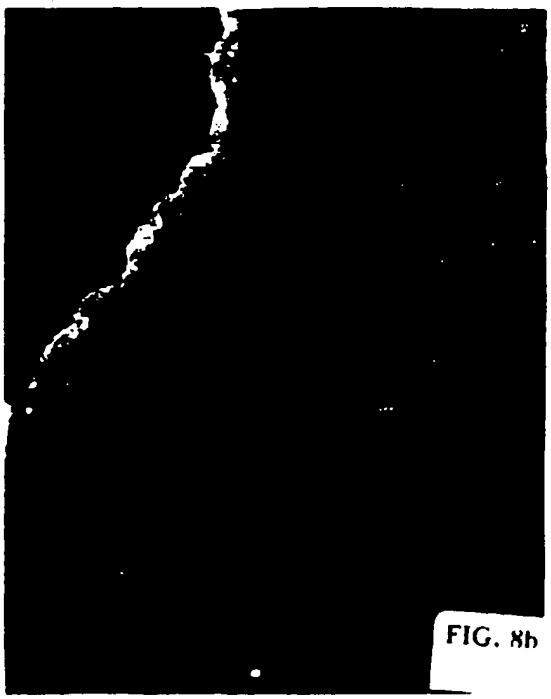

FIG. 8b

FIG. 8c

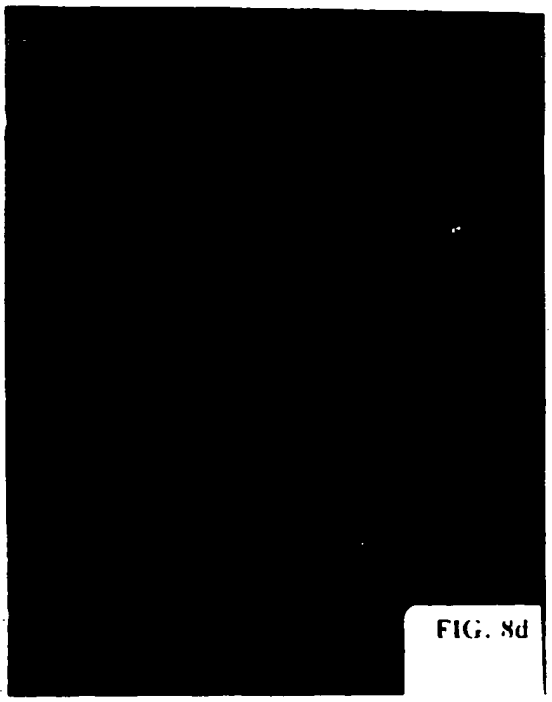

FIG. 8d

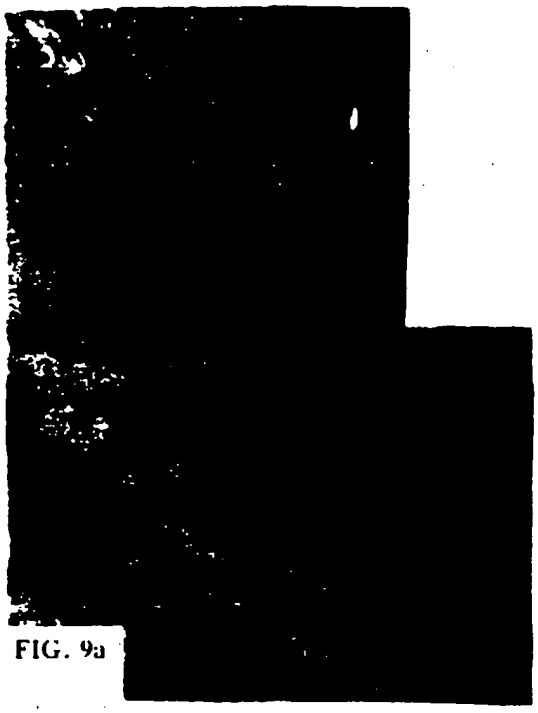

FIG. 9a

FIG. 9b

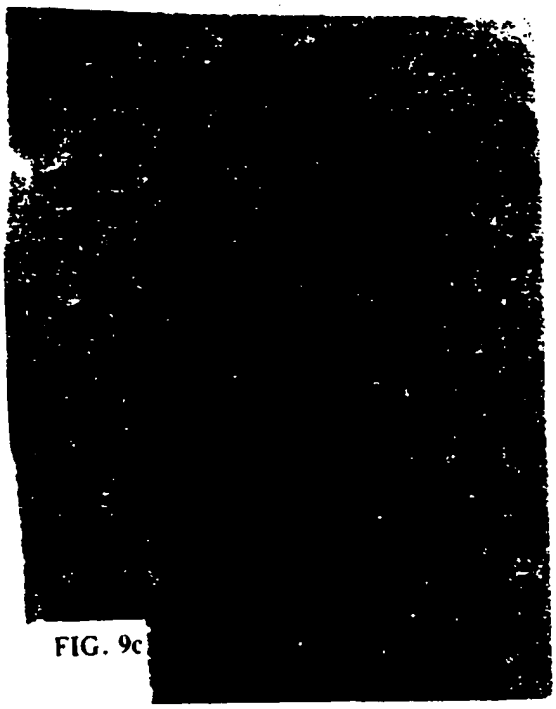

FIG. 9c

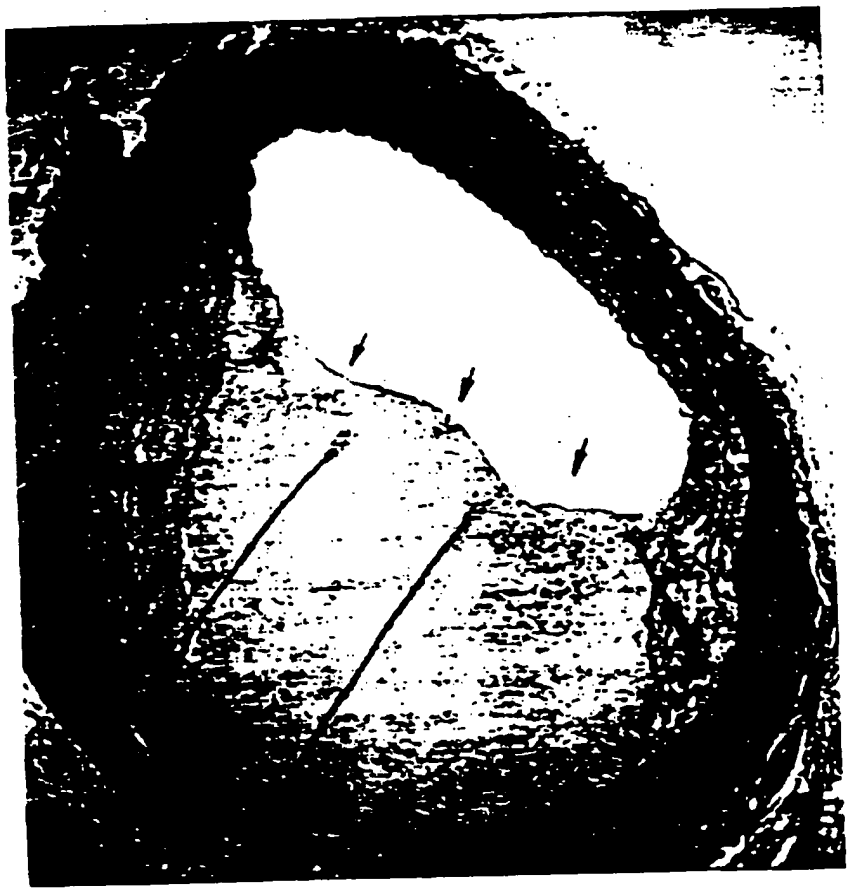

FIG. 10

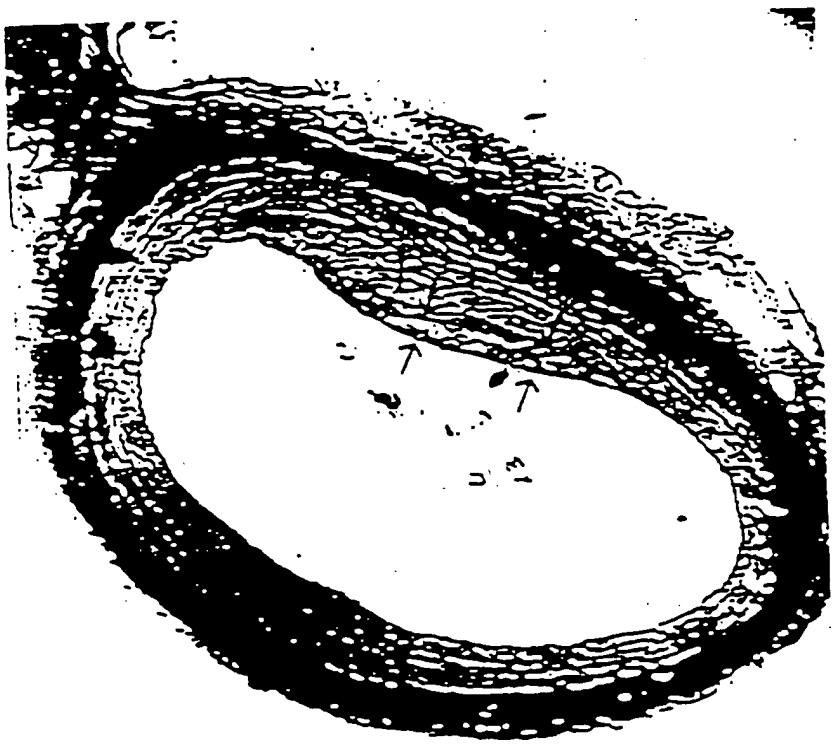

FIG. 11

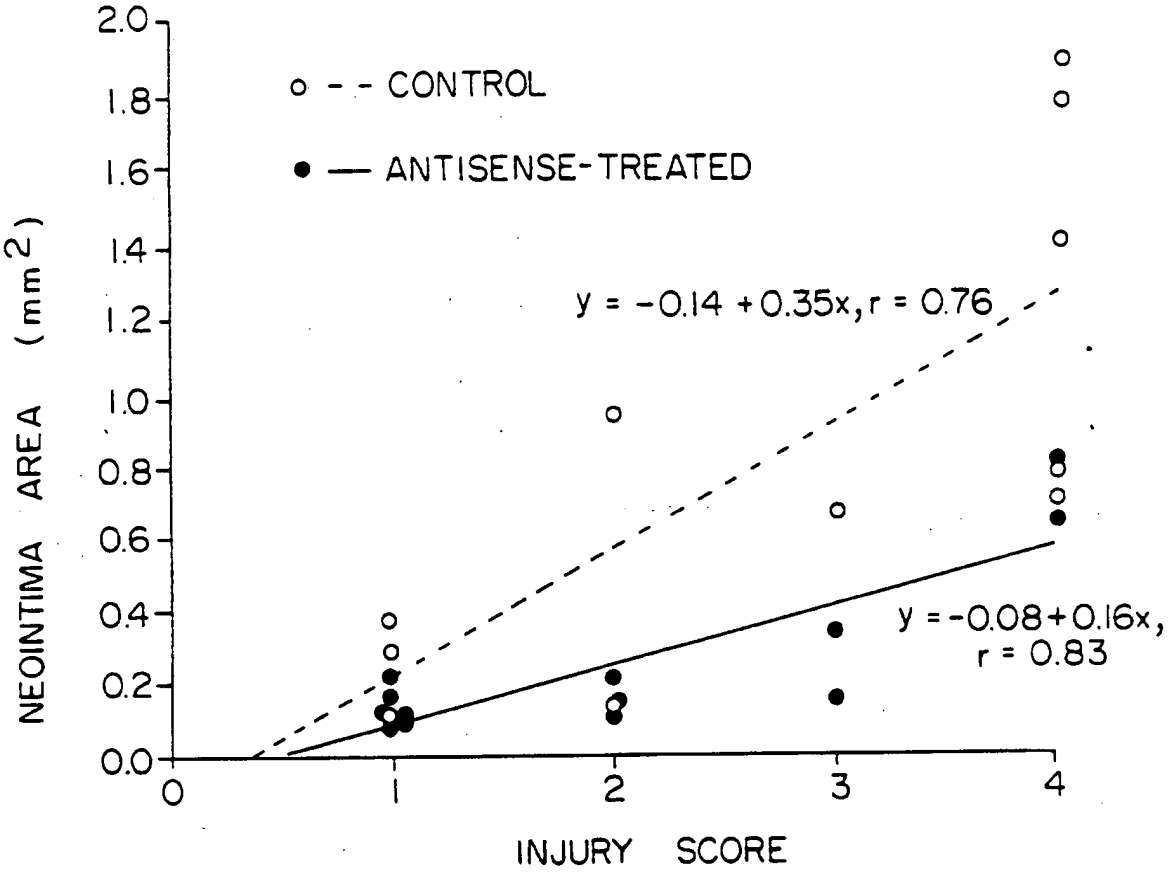
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NEOINTIMA AREA (mm2)
o - - CONTROL
● — ANTISENSE-TREATED
y = -0.14 + 0.35x, r = 0.76
y = -0.08 + 0.16x,
r = 0.83
0
1
2
3
4
INJURY SCORE

FIG. 12

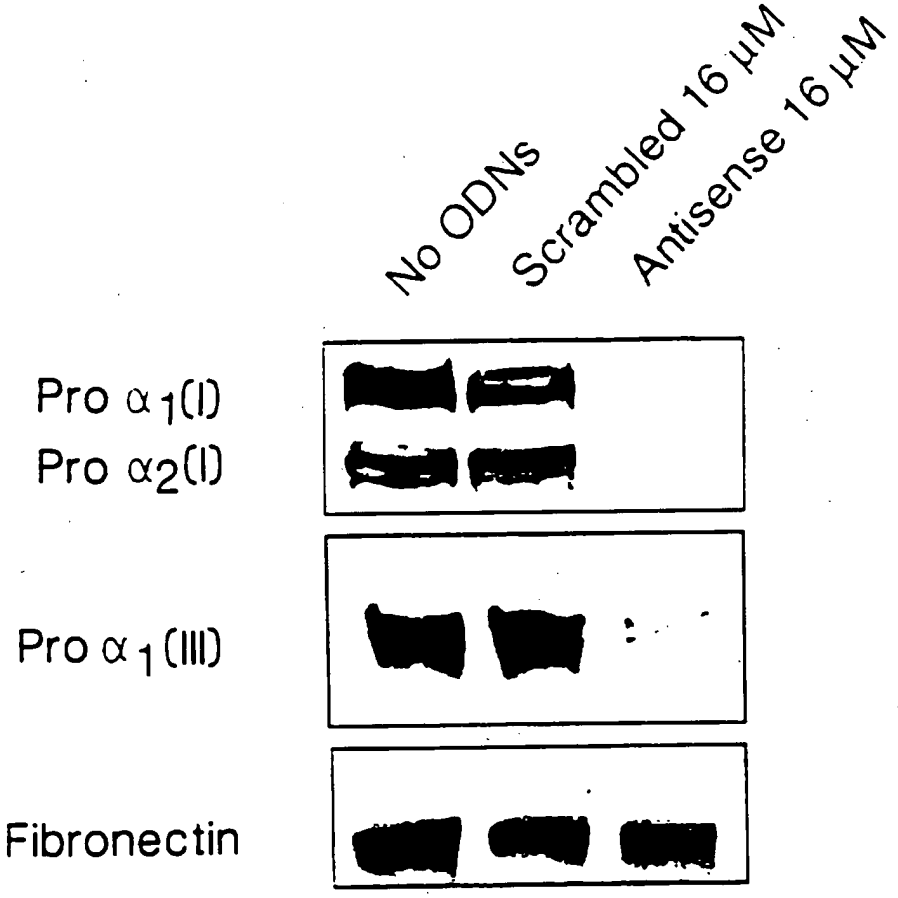
No ODNs
Scrambled 16 μM
Antisense 16 μM
Pro α1(I)
Pro α2(I)
Pro α1(III)
Fibronectin

FIG. 13

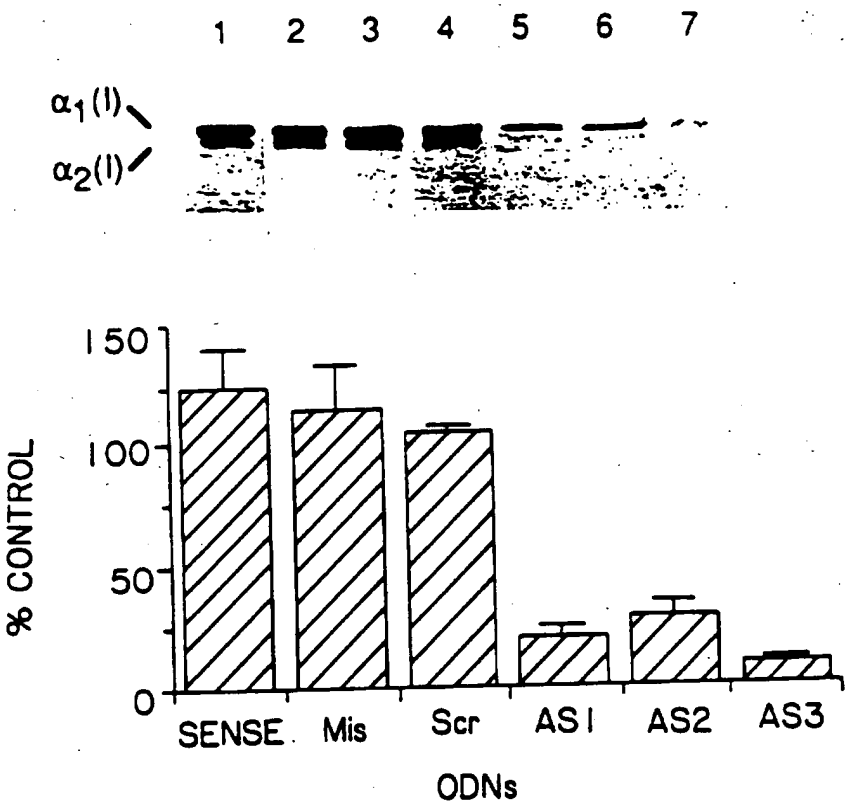
1 2 3 4 5 6 7
α1(I)
α2(I)
150
100
50
0
% CONTROL
SENSE
Mis
Scr
AS1
AS2
AS3
ODNs

FIG. 14

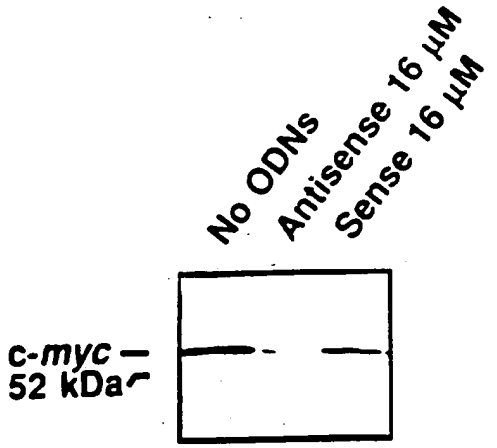
No ODNs
Antisense 16 μM
Sense 16 μM
c-myc —
52 kDa

FIG. 15

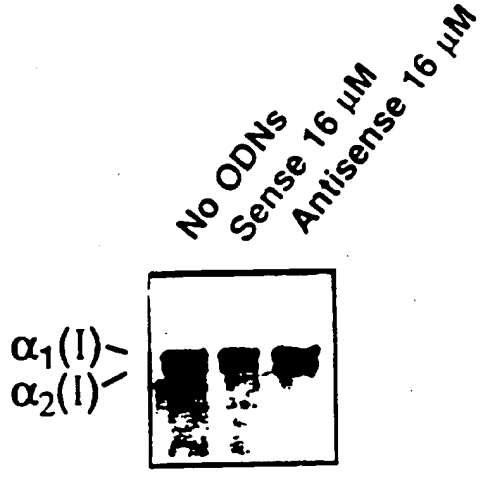
No ODNs
Sense 16 μM
Antisense 16 μM
α1(I)
α2(I)

FIG. 17

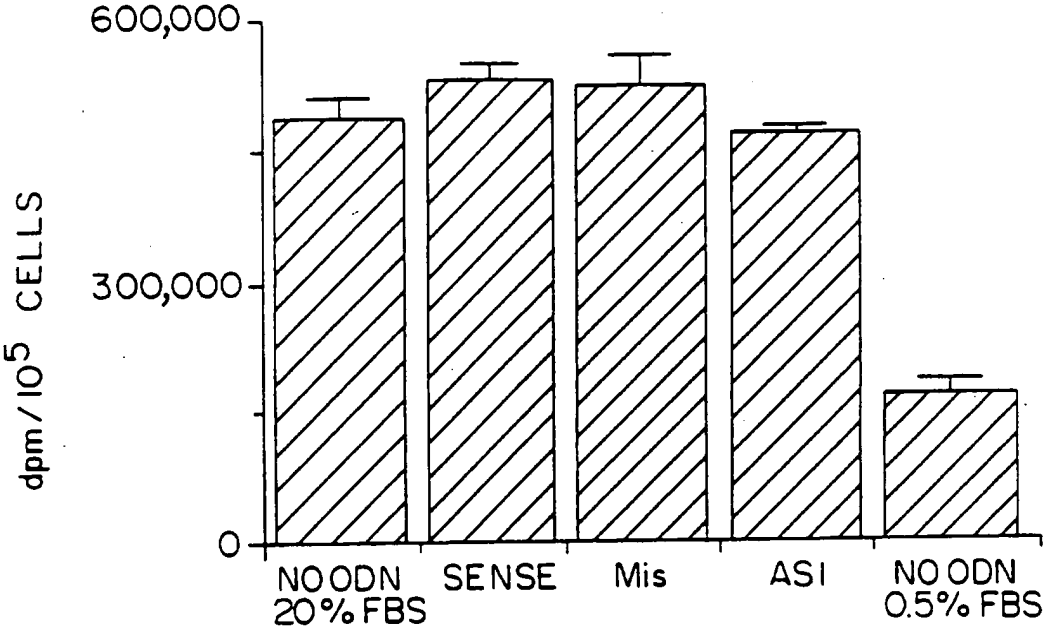
600,000
300,000
0
dpm/10^5 CELLS
NO ODN 20% FBS
SENSE
Mis
ASI
NO ODN 0.5% FBS

FIG. 16

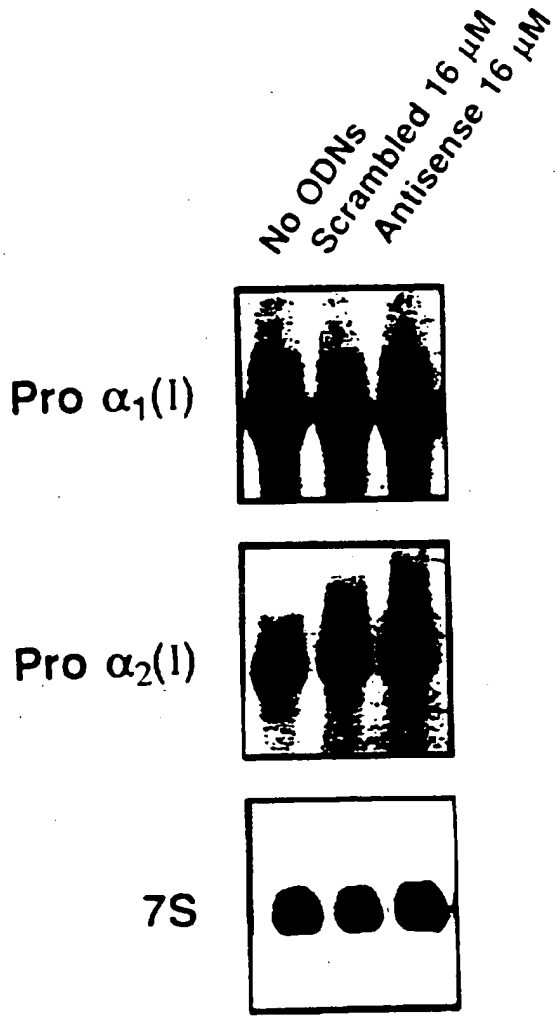


FIG. 18

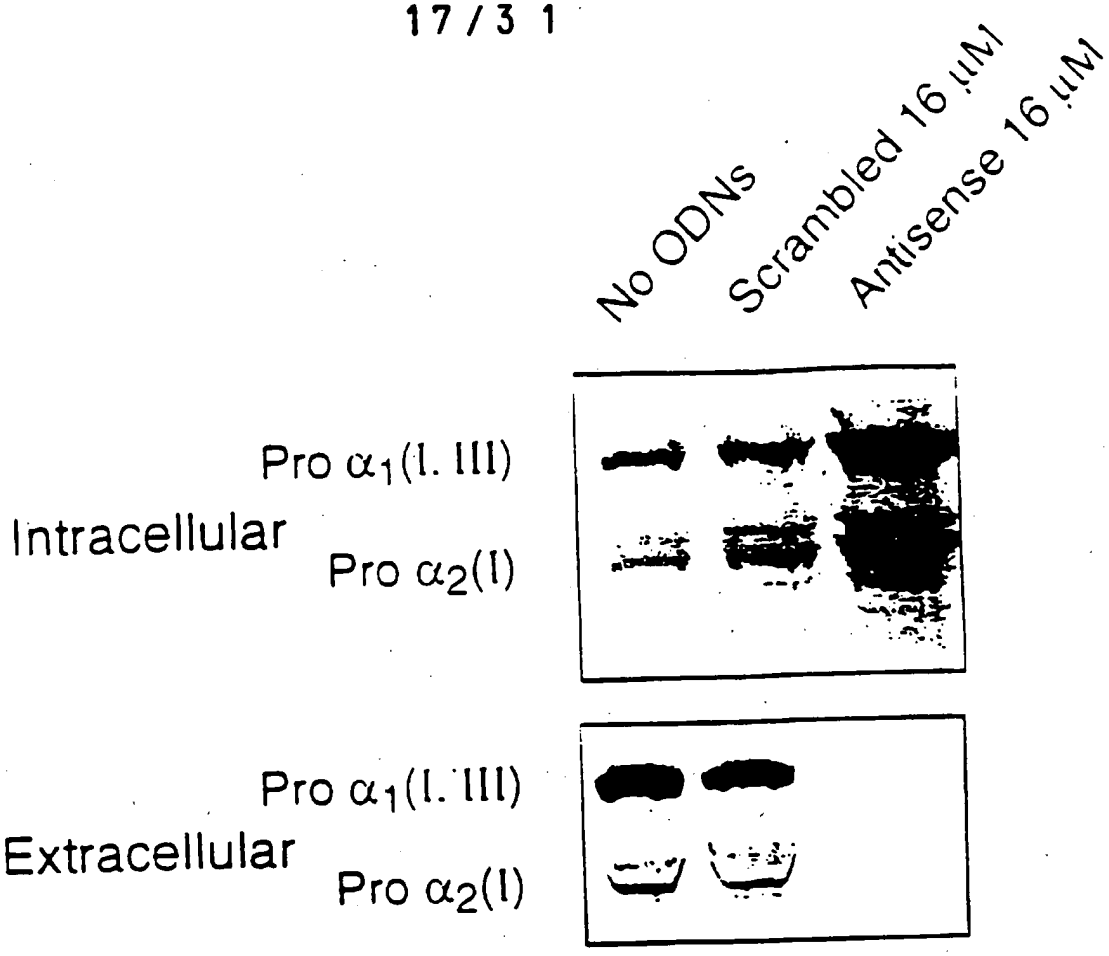
No ODNs
Scrambled 16 μM
Antisense 16 μM
Pro α1(I. III)
Intracellular
Pro α2(I)
Pro α1(I. III)
Extracellular
Pro α2(I)

FIG. 19

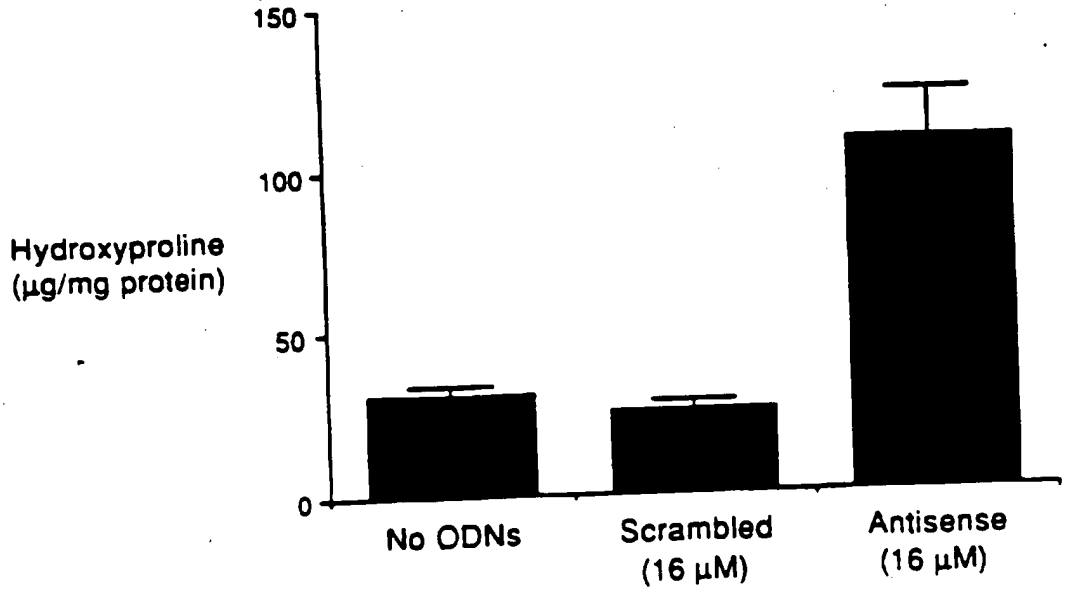
150
100
50
0
Hydroxyproline
(μg/mg protein)
No ODNs
Scrambled
(16 μM)
Antisense
(16 μM)

FIG. 20

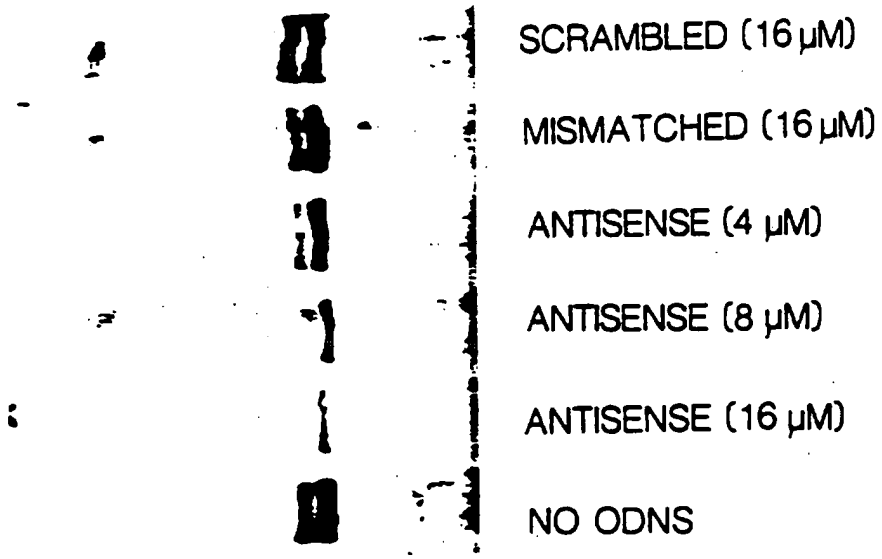
SCRAMBLED (16 µM)
MISMATCHED (16 µM)
ANTISENSE (4 µM)
ANTISENSE (8 µM)
ANTISENSE (16 µM)
NO ODNS

FIG. 21

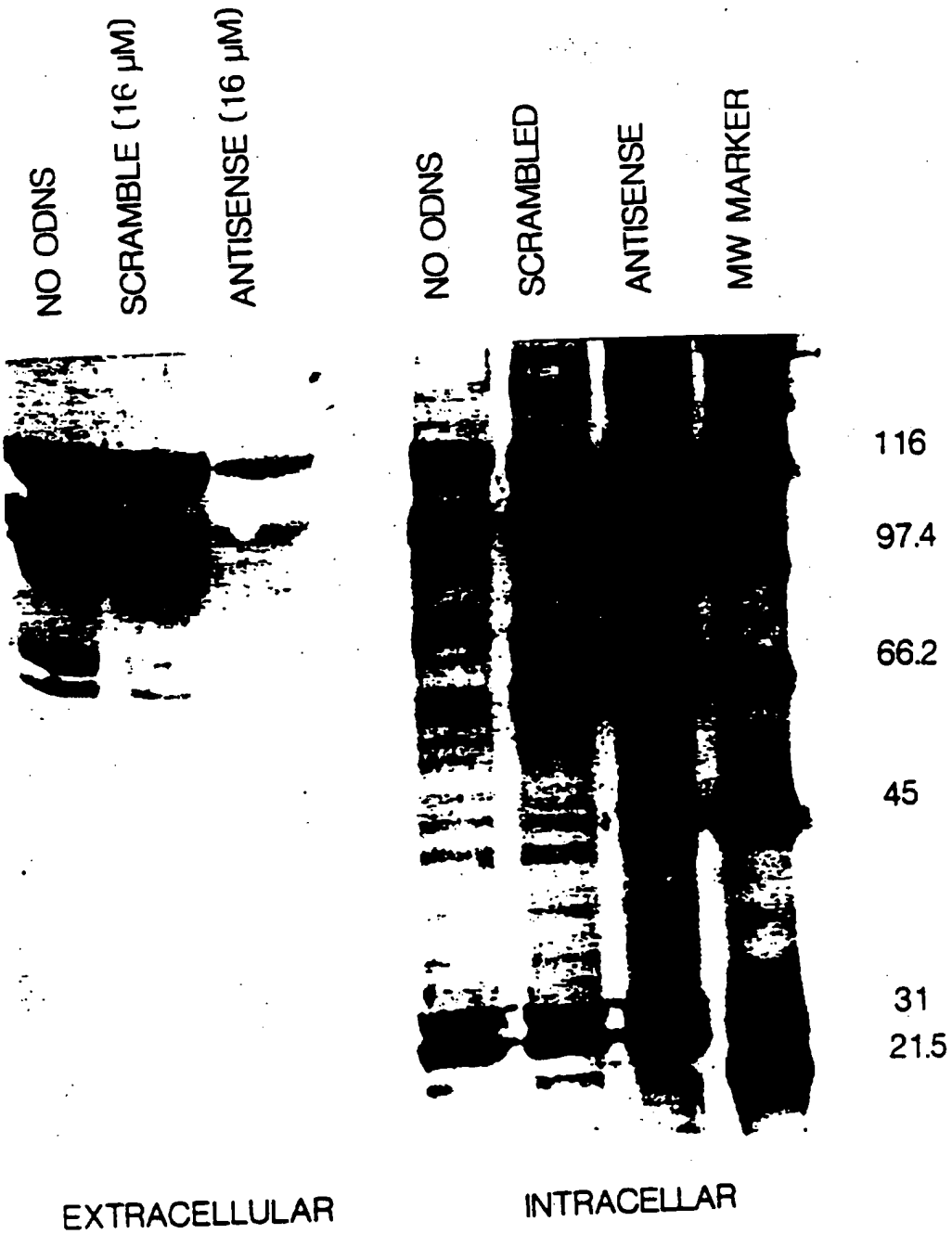
NO ODNS
SCRAMBLE (16 μM)
ANTISENSE (16 μM)
NO ODNS
SCRAMBLED
ANTISENSE
MW MARKER
116
97.4
66.2
45
31
21.5
EXTRACELLULAR
INTRACELLAR

FIG. 22

Temp.(°C)
No ODNs
Scrambled 16 μM
Antisense 16 μM
37 41 43
37 41 43
37 41 43
α1 (I) —
α2 (I) —

FIG. 23

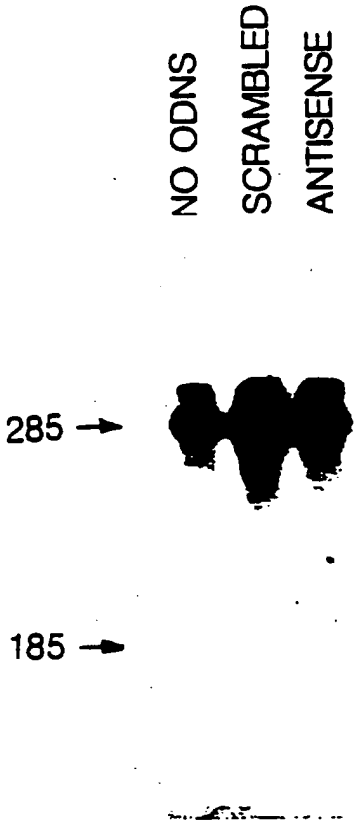
NO ODNS
SCRAMBLED
ANTISENSE
285
185

FIG. 24

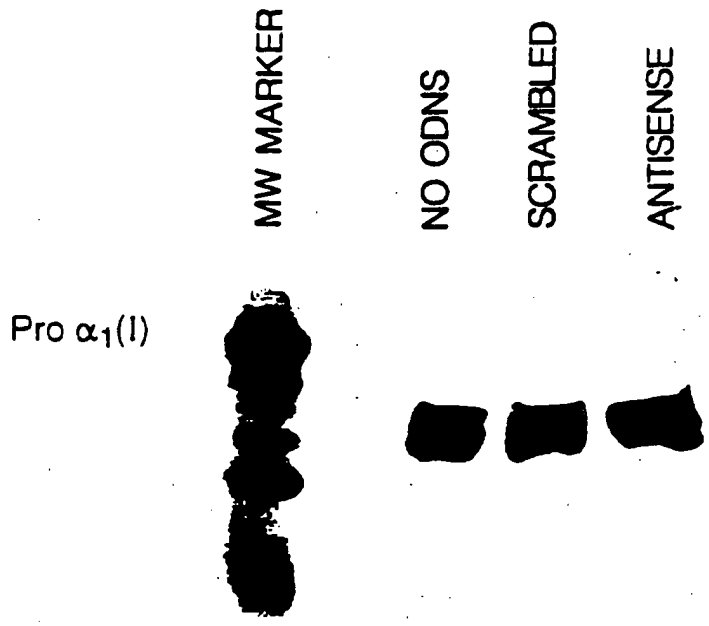
MW MARKER
NO ODNS
SCRAMBLED
ANTISENSE
Pro $\alpha_1$(I)

FIG. 25

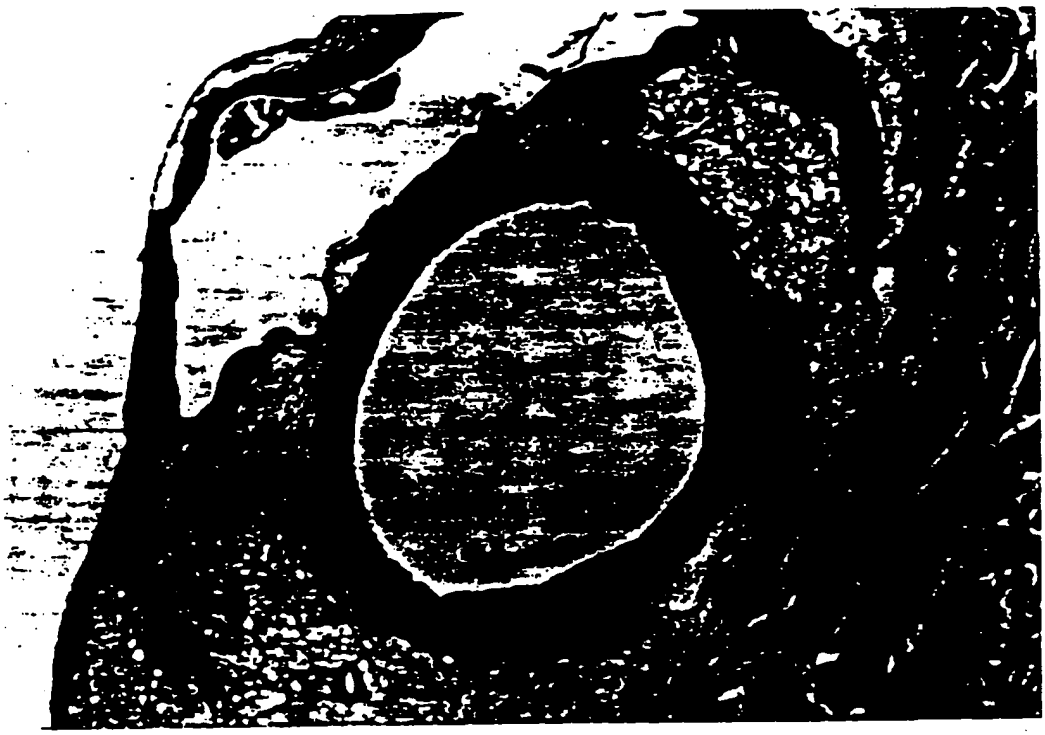

FIG. 26

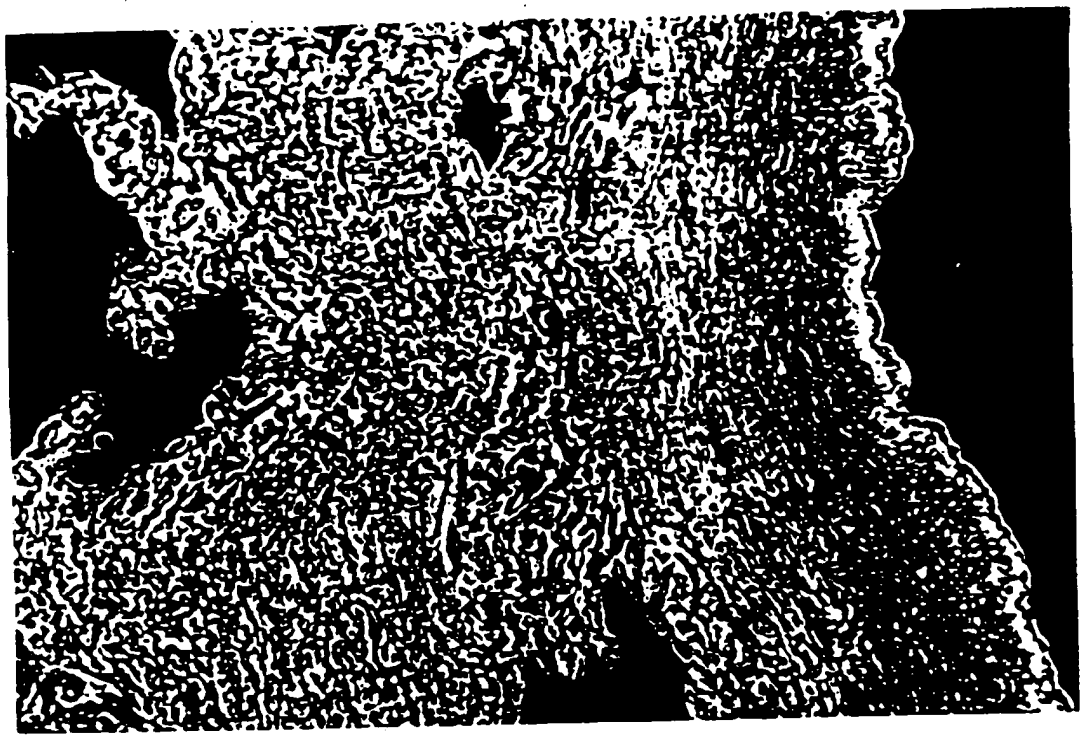

FIG. 27 A

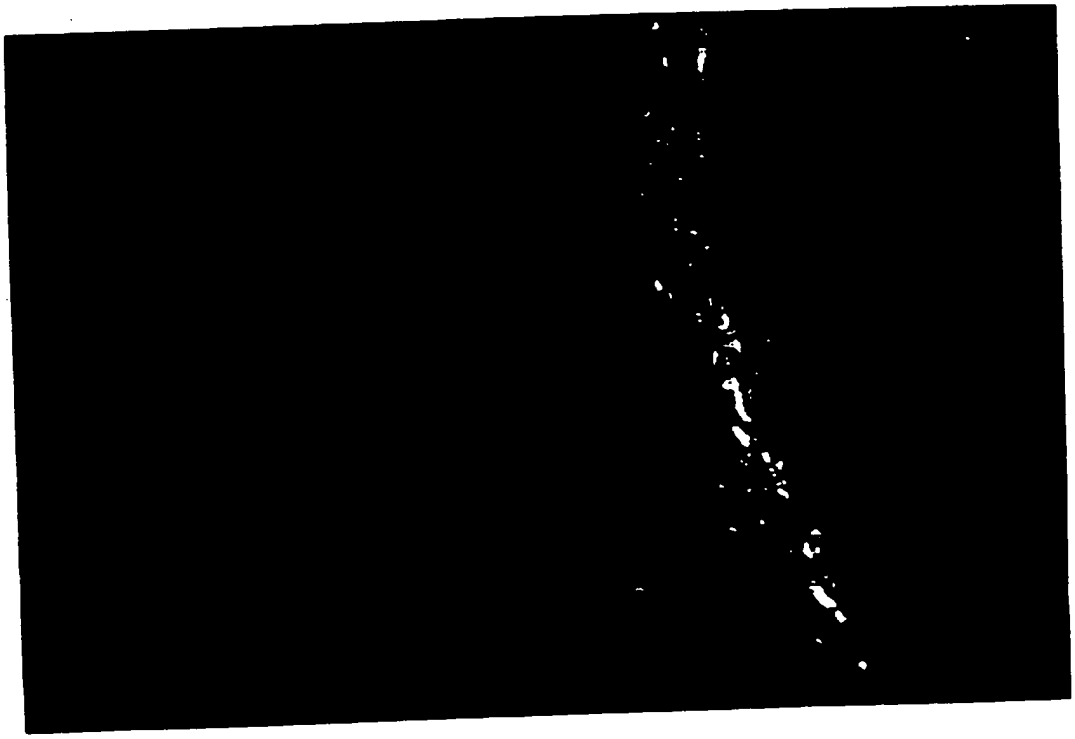

FIG. 27 B

FIG. 27 C

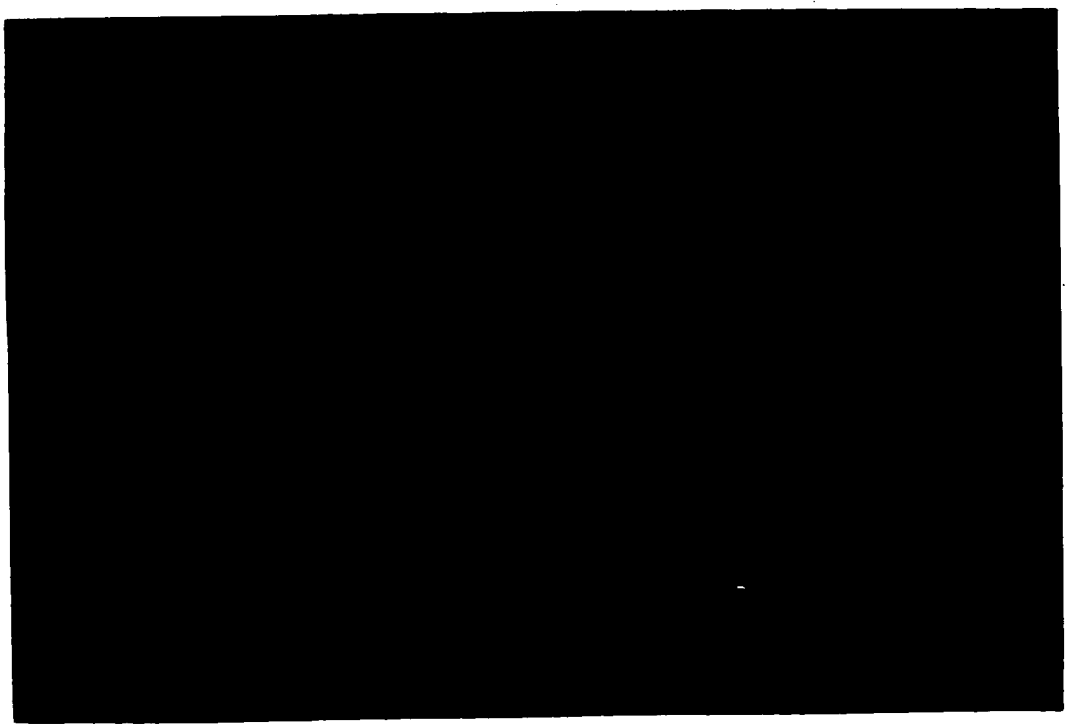

FIG. 27 D

FIG. 27 E

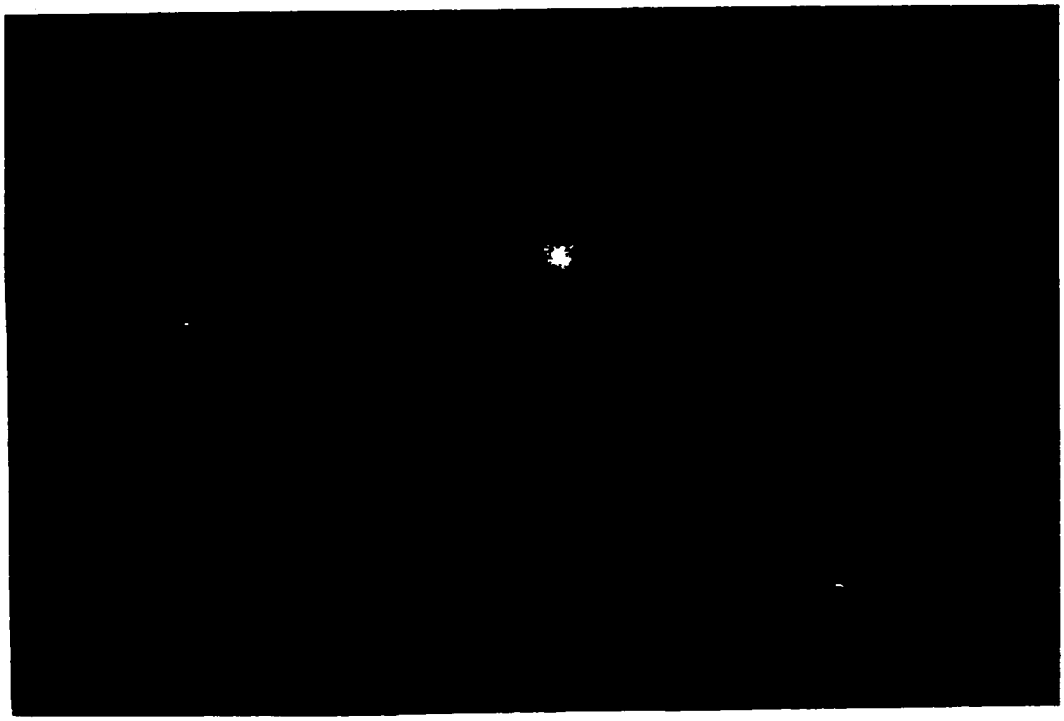

FIG. 27 F

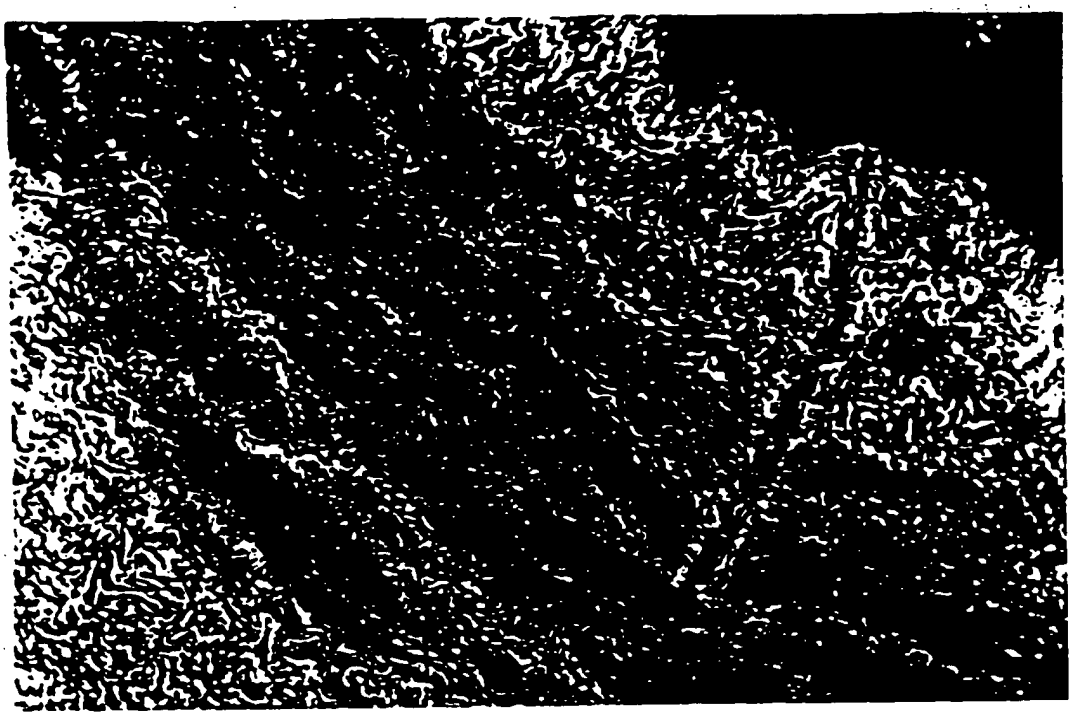

FIG. 28 A

FIG. 28 B

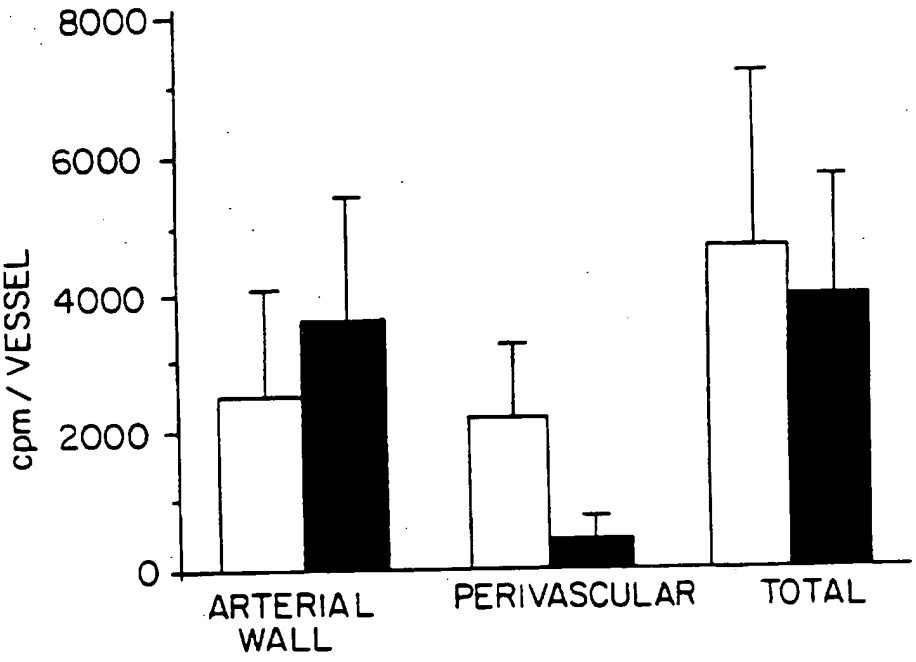
8000
6000
4000
2000
0
cpm / VESSEL
ARTERIAL WALL
PERIVASCULAR
TOTAL

FIG. 29

FIG. 30 A

FIG. 30 B

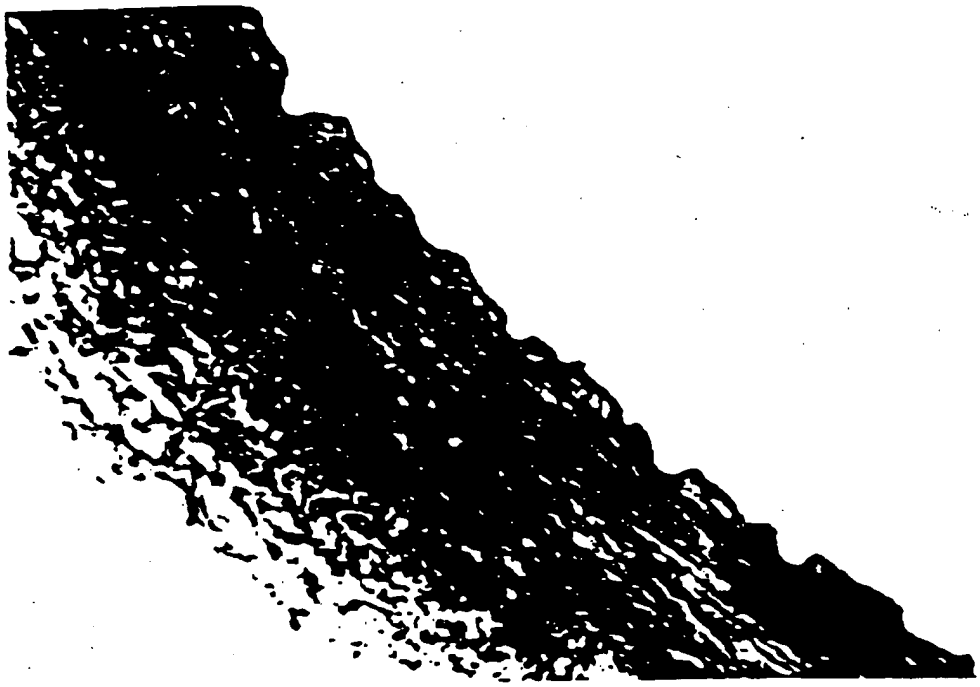

FIG. 30 C

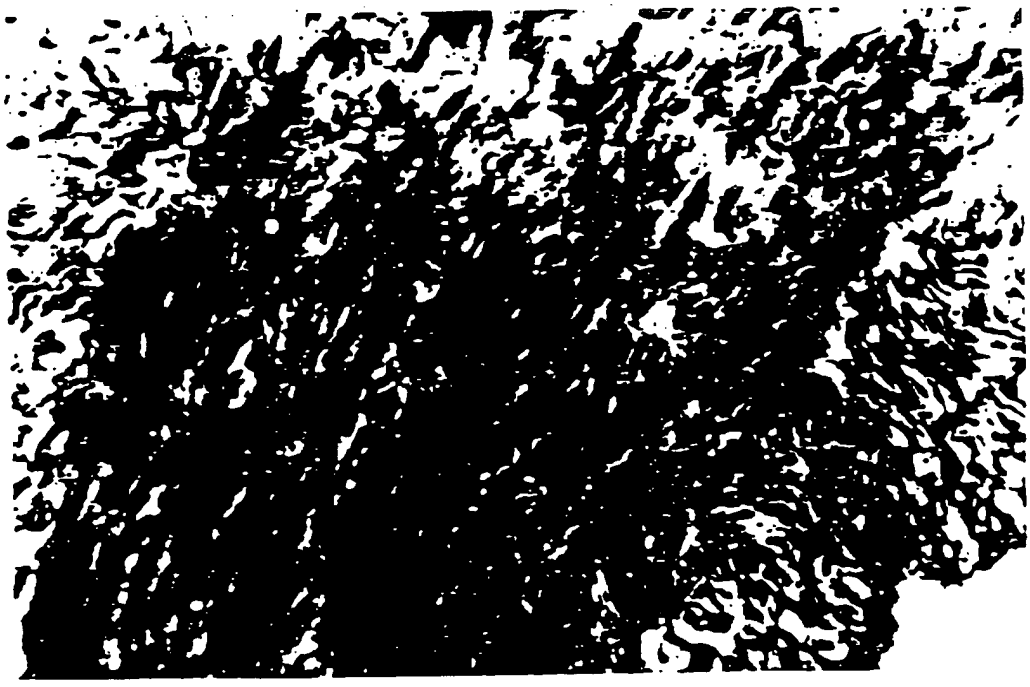

FIG. 30 D


FIG. 31

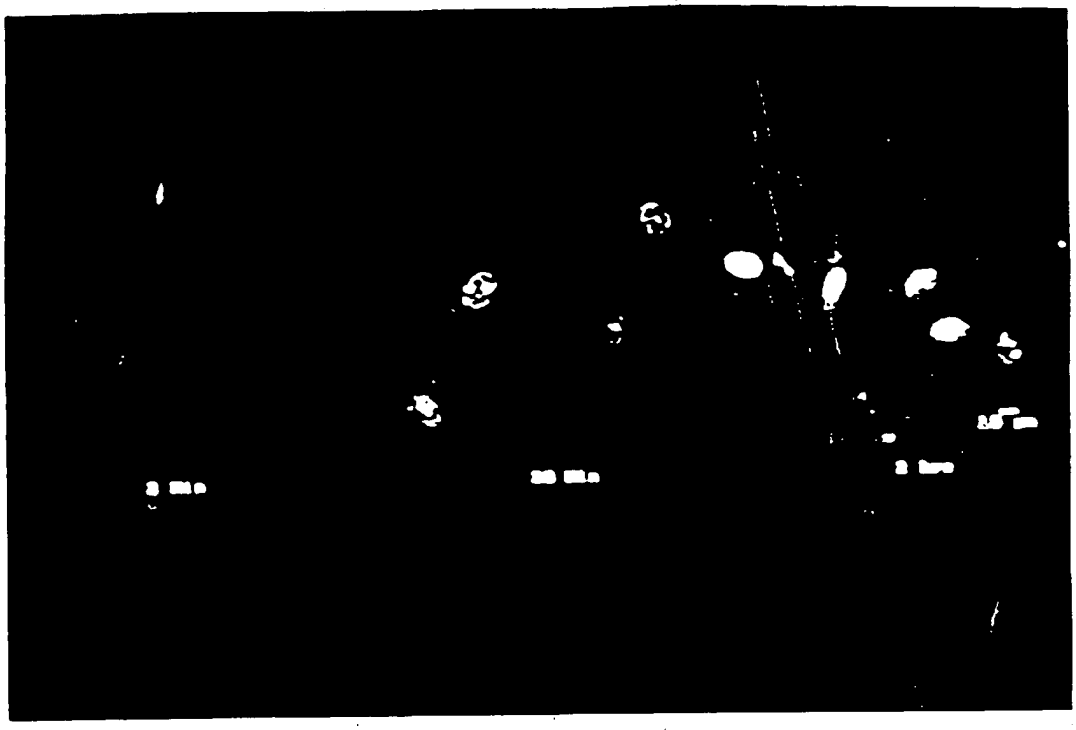

FIG. 32

FIG. 33

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05334

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00
US CL :514/44; 424/93.21
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 94/15646 A1 (THOMAS JEFFERSON UNIVERSITY) 21 July 1994, see entire document. | 1-59 |
| Y, P | WO 95/10305 A1 (THOMAS JEFFERSON UNIVERSITY) 20 April 1995, see entire document. | 1-59 |
| Y | SHI et al. Transcatheter Delivery of c-myc Antisense Oligomers Reduces Neointimal Formation in a Porcine Model of Coronary Artery Balloon Injury. Circulation. August 1994, Vol.90, pages 944-951, see entire document. | 1-59 |
| Y | SHI et al. Inhibition of Type I Collagen Synthesis in Vascular Smooth Muscle Cells by C-myc Antisense Oligomers. Circulation. November 1994, Vol.90, page I-147, abstract no. 0787, see entire document. | 1-59 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search
28 JUNE 1996

Date of mailing of the international search report
15 JUL 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05334

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | SHI et al. Regulation of Extracellular Matrix Synthesis by Antisense Oligomers Targeting the C-myc Proto-Oncogene. Circulation. November 1994, Vol.90, page I514, abstract no. 2767, see entire document. | 1-59 |
| Y | SHI et al. Safety and Efficacy of Transcatheter Delivery of C-myc Antisense Oligomers in the Coronary Vasculature. Circulation. November 1994, Vol.90, page I393, abstract no. 2110, see entire document. | 1-59 |
| Y | FARD et al. Mechanisms of Neointimal Reduction After Transcatheter Delivery of C-myc Antisense Oligomers. Circulation. November 1994, Vol.90, page I191, abstract no. 1022, see entire document. | 1-59 |
| Y | SHI et al. C-myc Antisense Oligomers Reduce Neointima Formation in Porcine Coronary Arteries. Journal of the American College of Cardiology. March 1994, Special Issue, page 395A, abstract no. 798-5, see entire document. | 1-59 |
| Y, P | O'BRIEN et al. Inhibition of Cell Proliferation by C-myc Antisense Oligomers Following Surgical Revascularization. Circulation. November 1995, Vol.92, Suppl.8, page I297, abstract no. 1414, see entire document. | 1-59 |
| Y | SHI et al. Downregulation of c-myc Expression by Antisense Oligonucleotides Inhibits Proliferation of Human Smooth Muscle Cells. Circulation. 1993, Vol.88, pages 1190-1195, see entire document. | 1-59 |